Studies on potential biological indicator organisms for low temperature steam and formaldehyde (LTSF) sterilization

Chinyanganya, Farai W.

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STUDIES ON POTENTIAL BIOLOGICAL INDICATOR ORGANISMS FOR LOW TEMPERATURE STEAM AND FORMALDEHYDE (LTSF) STERILIZATION

Submitted by Farai W. Chinyanganya

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Thesis

This research was carried out in the School of Pharmacy and Pharmacology of the University of Bath under the supervision of Dr. C.J. Soper, B.Pharm., MSc., PhD., MRPharmS and Dr. D.J.G. Davies, MSc., PhD., FRPharmS.

ProQuest LLC
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P.O. Box 1346
Ann Arbor, MI 48106-1346

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Dedicated to my family and wife, Imma

Zviroto zvazuro, ichokwadi chanhasi.

Zviroto zvanhasi, ichokwadi chamangwana.

(Shona)

Dreams of yesterday, are the reality of today.

Dreams of today will become the reality of tomorrow

(English)

SUMMARY

This thesis reports on studies aimed at selecting a suitable biological indicator organism for Low Temperature Steam and Formaldehyde (LTSF) sterilization.

The Introduction defines sterilization, summarises principles of sterilization and reviews various sterilization methods and their validation. The role of Biological indicators in validating and monitoring LTSF sterilization is discussed. A summary of the kinetics of bacterial spore inactivation is included together with an account of factors that influence bacterial spore resistance to lethal treatments. The introduction also reviews the history of formaldehyde as a bactericide and the principles and development of the LTSF sterilization process.

The Experimental section is divided into five chapters. The first experimental chapter desribes experiments which compare growth and sporulation of selected Bacillus species on two chemically defined media (CDM).

Experiments described in Chapter 4 compare resistance of the selected spores to moist heat at 110°C. The influence of the CDM composition on bacterial spore resistance is examined and a suitable medium for the production of spores for LTSF biological indicators recommended.

Experiments reported in Chapter 5 compare the resistance of selected Bacillus spores to 0.5% w/v aqueous formaldehyde solution. The effect of temperature, and of storage of aqueous spore suspensions, on resistance of the spores is investigated.

Chapter 6 describes modifications carried out to convert a commercial LTSF sterilizer to an experimental test apparatus suitable for investigating spore resistance at conditions likely to be encountered in commercial LTSF protocols.

Chapter 7 reports on the effect of temperature and formaldehyde concentration on the inactivation of Bacillus stearothermophilus NCIB 8224 spores by LTSF using the modified sterilizer. The data indicate a number of basic faults in the commercial sterilizer and the need to redesign the equipment.

Chapter 8 contains a concluding discussion of the investigations reported in this thesis together with suggestions for future work.

CONTENTS

Page

Page

Page

Page

Page

Page

ORIGIN AND SCOPE

There is a need for drugs, biological products and devices used in the health service to be adequately sterilized and maintained in a sterile state to avoid possibilities of infections arising from their usage. There are a number of methods of sterilization in current use which are adequate for the majority of materials. The development and widespread use of high technology thermolabile equipment such as artificial pacemakers, sigmoidoscopes and electronic apparatus, has presented new challenges to sterilization technology and still remains a problem in the health services.

Low temperature steam and formaldehyde sterilization (LTSF) and ethylene oxide sterilization (EO) are the commonly used gaseous methods to sterilize heat-sensitive materials. LTSF has many advantages over EO sterilization. However, from trends already seen in international literature, it is evident that the use of EO sterilization will continue. In spite of the obvious advantages of LTSF including operator safety, lower residual levels and its non-explosive nature, investment in equipment and research has been in favour of EO sterilization. There is uncertainty and scepticism as to the efficacy of the LTSF process and this could be due to the lack of an established protocol that can be accurately monitored. Consequently the use of LTSF in the UK and North Western European countries has been restricted to hospitals.

The present limited use of LTSF could be a consequence of the lack of reliable commercial equipment and the lack of research, for example, to develop internationally accepted biological indicators necessary to monitor this multiparameter process. There is also paucity of detailed information on the influence of the process parameters on sterilization, which is necessary for LTSF sterilization and this could have hampered assessment of different commercial protocols. Recently there have been attempts to stimulate research related to LTSF sterilization and in the UK, the Department of Health is actively involved in funding various research projects relating to the LTSF process.

The need for more research in this field takes on an increasing importance with the recent emergence of Acquired Immune Deficiency Syndrome (AIDS) since increasing numbers of these patients can develop life threatening opportunistic mycobacterial infections involving the lungs and the intestines. Diagnosis of such cases frequently involves the use of thermolabile bronchoscopes and sigmoidoscopes which need thorough sterilization to reduce the risk of cross-contamination.

This thesis reports on investigations carried out to characterize potential biological indicator organisms for the LTSF process to provide more information about their behaviour as monitors at LTSF and related conditions.

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

1.1 PRINCIPLES OF STERILIZATION

Sterilization is the process designed to produce a sterile state or sterility. Sterility is defined as the absence of all viable life forms. Sterility and sterilization are both abstract terms describing a negative concept which cannot be shown to have been achieved but can be approached with an increasing probability of success as the sterilization process is improved. Because microorganisms die exponentially or logarithmically during a sterilization process, the level of sterility is best expressed on a probabilistic basis. There is always a finite chance of leaving some live microorganisms intact in the system. Sterilization processes are therefore designed to minimise the probability of leaving survivors. For implantable devices and injectable drugs, the probability of a nonsterile unit (PNSU) should be less than one in a million units (1). The way in which micro-organisms die makes it impossible to practically attain sterility. In practice, sterilization has often been taken as a process by which all viable life forms can be assumed to have been killed.

With all articles to be sterilized there is often the chance that the sterilizing process will have a detrimental effect. During the design of sterilization protocols, a compromise is often reached between the most effective sterilization procedure and one

that will not have a significant adverse effect on the material being sterilized. The most effective sterilizing procedure is the one that reduces the area of calculated risk to a minimum (2).

1.2 STERILIZATION METHODS

Items can be sterilized by exposure to physical or chemical agents for a defined period. The commonly used methods include moist and dry heat, radiations, toxic gases and the combination of sublethal heat with bacterial chemicals. In addition, fluids and gases can be sterilized by the physical removal of microorganisms by filtration. While most of these methods are adequate for the vast majority of medical items and preparations, some products are sensitive to the process, e.g. heat sensitive materials. Toxic bactericidal gases are used to sterilize such thermolabile items. The only gases in current use are ethylene oxide and formaldehyde gas with low temperature steam.

1.3 KINETICS OF BACTERIAL SPORE INACTIVATION

To a first approximation populations of microorganisms exposed to a lethal treatment often die or lose viability logarithmically, the fraction of survivors decreasing exponentially with time. Survivor curves are often used to express graphically the microbial inactivation when the surviving fraction (N_t/N_o) on a logarithmic scale, is plotted against the exposure time, t, on a linear scale. The survivor curves often take one of the four forms

shown in Fig. 1. For each survivor curve type, the shape is independent of the initial number of microorganisms (bioburden).

Type A curves show a linear exponential pattern with a constant fraction of the population inactivated per unit time. This curve can be described by the mathematical expression for first order chemical reaction kinetics (3) as

$$\frac{N_t}{N_o} = e^{-kt} \qquad \text{...eq. 1}$$

where N_o = initial viable count

N_t = viable count at time t

t = exposure time

k = inactivation rate constant

The inactivation rate constant, k, can be calculated from the slope of the survivor curve and is a measure of spore sensitivity to the inactivating treatment. The higher the k value , the more sensitive is the organism to the treatment.

Type B curves exhibit initial shoulders or lag periods in killing before assuming a linear form. This has been shown for example when some highly resistant spores were exposed to dry heat (4). The following relationship was proposed for type B curves obtained during heat inactivation of spores equilibrated to various water activities (a_w) (5).

$$(\log N_o - \log N)^a = kt + c \qquad \text{... eq. 2}$$

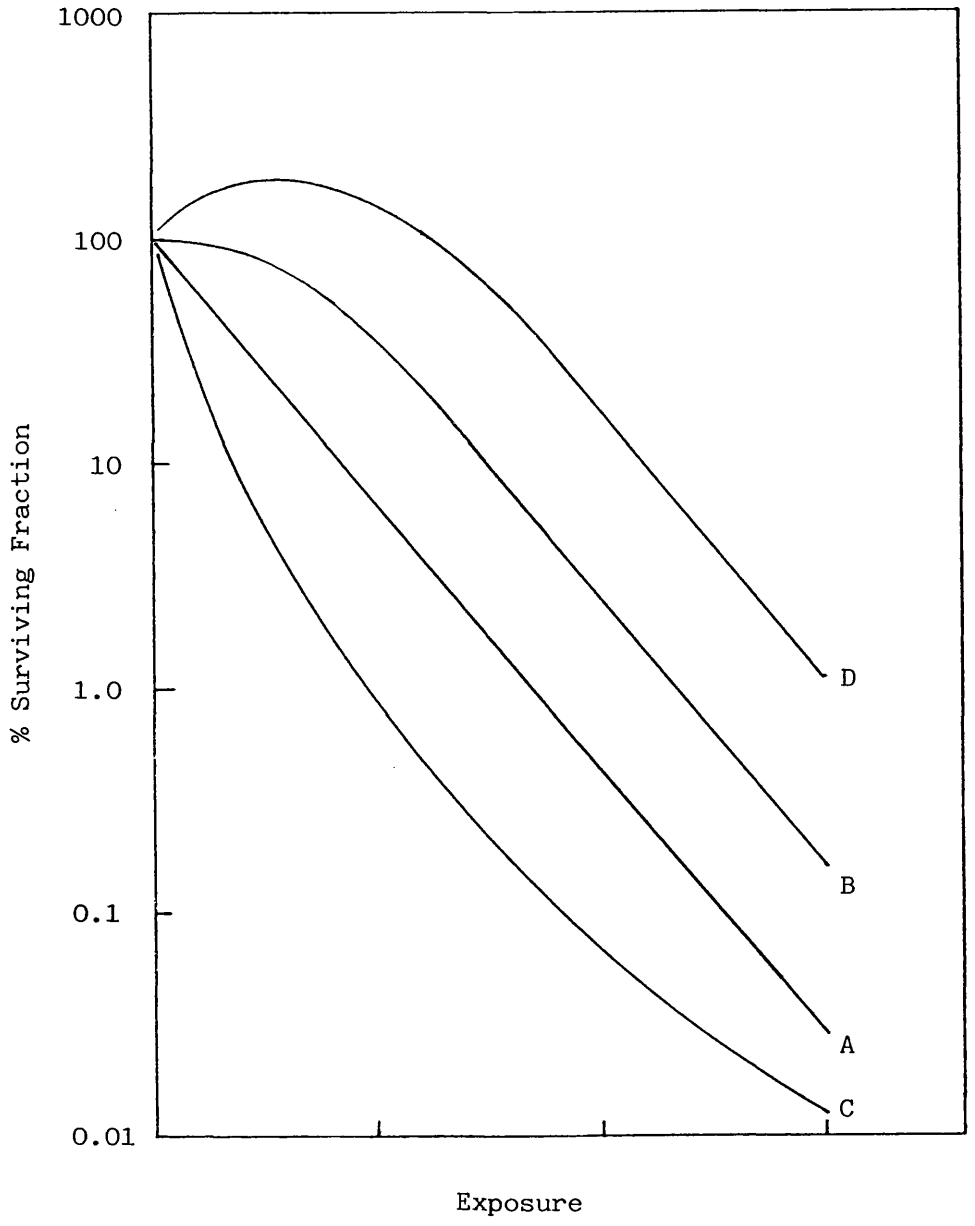


Fig. 1. Types of survivor curves for the inactivation of bacterial spores by lethal treatments.

where the exponent 'a' would linearise the shouldered curve. When 'a' = 1, the curve is log-linear. The value of 'k' and the equation constant c, can be determined from the slope and the intercept of the linearised survivor curve respectively and 'a' is the reciprocal slope of a plot of $\log(\log N_o - \log N)$ against log time (6).

The type C curve exhibits a decreasing death rate with time. In some heat inactivation experiments this curve can be biphasic with a steeper initial fall in viability followed by a distinct phase with a shallower fall. The presence of a more resistant fraction of the spore population or the disappearance of lethal agent at prolonged exposure periods e.g. formaldehyde in LTSF, can result in a type C curve. It has been claimed that all three types of the survivor curves are modifications of a basic sigmoid curve, where the initial lag may be too short to be detectable, and recovery of organisms at low survivor levels may be too erratic to provide accurate determinations (7).

B. stearothermophilus spores of low germination index may be activated by heat and this shows on the survivor curve as an initial rise in viability due to activation of dormant spores followed by the killing of activated spores which may or may not be strictly log-linear (Type D) (9).

The intercept ratio (IR) was proposed to distinguish between the various survivor curve types without necessarily

examining the curves (9). This is defined by the relationship

$$IR = \log Y_o / \log N_o \qquad \text{... eq. 3}$$

where Y_o = zero-time intercept on the Y axis of the linear portion on the survivor curve

N_o = initial viability of spores at time, t_o

When Y_o value is greater than N_o value (IR > 1) the survivor curve will be either Type B or D. When Y_o value is less than the N_o value (IR $\leq$ 1) the survivor curve will be Type C. When IR = 1, the survivor curve is Type A.

1.4 MICROBIAL DEATH KINETIC TERMS

A number of mathematical terms are often used to express bacterial spore resistance. These expressions are based on the assumption of semilogarithmic death patterns and first order kinetics.

1.4.1 Decimal Reduction Time (D-Value)

This is the time in minutes for moist heat, dry heat and chemical inactivation or the absorbed dose for ionizing radiation, required to effect a 90% reduction in the microbial population or a one log cycle reduction on the survivor curve as illustrated in Fig. 2 (10). The D-value can be expressed mathematically as

$$D = \frac{t}{\log N_o - \log N_t} \qquad \text{...eq. 4}$$

where N_o = initial viable count at time t = 0

N_t = viable count at time t = t

t = exposure time

The temperature of inactivation to which the value corresponds is always shown as a subscript, i.e. D_{121} is the D-value at 121°C. For Types B, C, D survivor cuves, the D-value can be estimated from the linear portions of the curves. Numerically, the D-value is also the reciprocal of the inactivation rate constant k calculated from the relationship

$$k = \frac{2.303}{t} \log_{10} \frac{N_o}{N_t} \qquad \text{... eq. 5}$$

D-values can be used to compare sensitivities of different bacterial spore populations provided they all exhibit semilogarithmic survivor curves. They however, become inadequate when comparing resistance of spore populations that exhibit shoulders, activation and tailing on their survivor curves. IR values can be used to qualify the nature of quoted D-values whether they are from Type A survivor curves (IR = 1) or they are obtained from linear portions of Types B, C and D curves when IR is greater than 1 for Type B or D and less than 1 for Type C survivor curves.

1.4.2 Inactivation Rate Constant, k

The slope of the survivor curve is -k/2.303 where k is the inactivation rate constant. It has been mathematically described by equations 1 and 5.

1.4.3 Arrhenius Relationship

Assuming first order death kinetics, the temperature of inactivation and the inactivation rate constant k can be related by the Arrhenius relationship as

$$k = A_e^{-Ea/RT} \qquad \text{... eq. 6}$$

where k = inactivation rate constant ($time^{-1}$)

A = frequency factor ($time^{-1}$)

E_a = activation energy of bacterial death

R = universal gas constant

T = Absolute temperature (K)

The equation can be expressed in the logarithmic form as:

$$\log k = \log A - Ea/2.303RT \qquad \text{... eq. 7}$$

The Arrhenius plot is obtained by plotting the inactivation rate constant k, on a log scale against the reciprocal of the corresponding Absolute temperature 1/T (K^{-}) on a linear scale. The

Activation Energy of the lethal process, Ea, can then be calculated from the slope of this plot which is equal to -Ea/2.303R (11).

1.4.4 Z-value

The Z-value, also termed the resistance value, is defined as the increase in temperature needed to reduce the D-value of an organism by 90% or one log-cycle on the $\log_{10}$ D-value against temperature curve. The more resistant the organism is, the larger the Z-value. The Z-value is a fundamental characteristic of any given species and is dependent on the lethal process employed.

1.4.5 Inactivation Factor (IF)

The Inactivation Factor (IF) is defined as the reduction in the numbers of a given organism by a defined sterilization process (Fig. 2). The IF provides the simplest method of calculating the probability of achieving sterility for any given initial survival level since there is no finite time within which all the organisms present can be assumed dead with 100% certainty. The IF value can be expressed in terms of the D-value for the organism as:

$$IF = 10^{t/D} \qquad \text{... eq. 8}$$

where D = Decimal Reduction Time (D-value)

t = time of exposure to the conditions

Assuming an initial viable count of 10^6 organisms and an exposure

time of t minutes resulted in an IF of 10^{12}, then the chance of obtaining an unsterile item will be one in a million, i.e. a 10^{-6} probability of an organism surviving the treatment (Fig. 2). The British Pharmacopoeia (1988) recommends sterilizing by autoclaving at 121°C for 15 minutes. Spores of Bacillus stearothermophilus NCTC 10007 are recommended by the British Pharmacopoeia protocol as suitable test organisms for sterilization by autoclaving. These spores have a D-value at 121°C of 1.5 minutes. Therefore autoclaving at 121°C for 15 minutes would have an IF of 10^{10} for a suspension of that organism. The IF values are often expressed as the exponent t/D only as in the use of the '6D' and '12D' concepts which specify that the processes are required to produce inactivation factors of 10^{6} or 10^{12} respectively (2).

1.4.6 Most Probable Effective Dose ($MPED_n$)

IF-values based on the knowledge of the D-value and exposure time can be accurately used to measure the total destructive power of a lethal process provided the survivor curve for that organism is log-linear over the entire exposure time range. For occasions when the survivor curves show shoulders (Type B) or tailing (Typc C), an extra holding time is often added as an allowance for the non-linearity. The food industry introduced the $MPED_n$ value (Fig. 2) defined as the most probable effective dose to achieve "n" decimal reductions in the number of organisms (2). This value is independent of the survivor curve shape but for linear survivor curves,

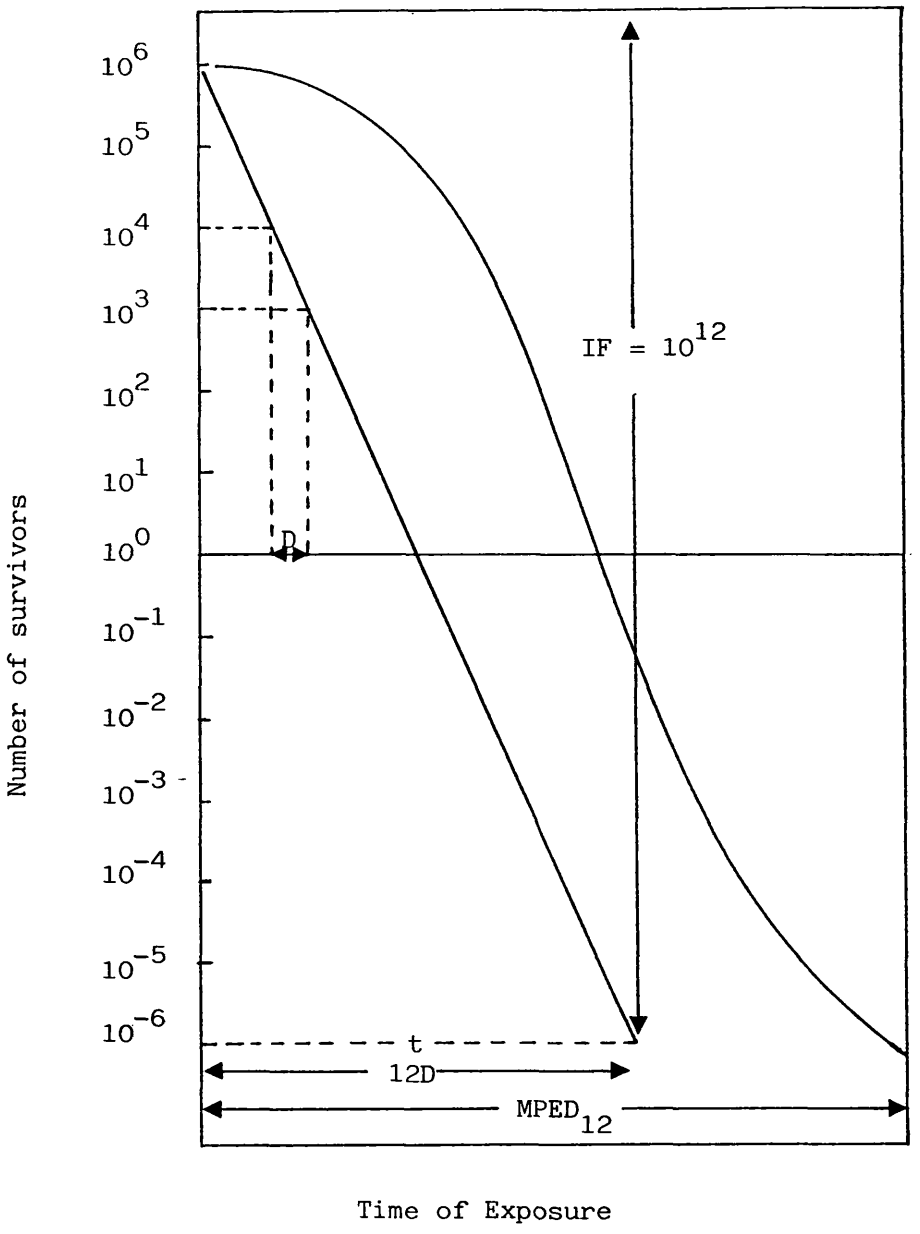


Fig. 2. Log-linear and non-log-linear survivor curves of numbers of survivors on A log scale against time, to show IF for time t, and 12D and $MPED_{12}$ from Soper, C.J. (2).

$$MEPD_n = nD \qquad \text{... eq. 9}$$

where D = D-value

n = IF exponent

1.4.7 F-value

The F-value is a "unit of lethality" and is defined as a measure of the total process lethality, equating heat treatment at any temperature to the time in minutes at a designated reference temperature required to produce the same lethality in a reference organism (2). The F-value is used in the food industry to compare the relative sterilizing capacities of different heat processes. Thus if a sterilization process has an F-value of 15, the sum of all lethal effects of the process is equivalent to the lethal effects of 15 minutes at the designated reference temperature (2).

1.4.8 F_o-value

The F-value compares lethal effects at different temperatures and is therefore dependent on the Z-value of the reference organism. Spores of B. stearothermophilus which have a Z-value of 10°C are often used as reference organisms at a reference temperature of 121°C. Under these conditions the F-value becomes the F_o-value. Thus a process with an F_o-value of 10 has the same lethality on B. stearothermophilus spores as heating at 121°C

for 10 minutes. The F_o-value can also be derived from the D_{121} value and the IF-value as

$$F_o = D_{121} (\log N_o - \log N_t) = D_{121} \log IF \qquad \ldots \text{ eq. 10}$$

where N_o = initial viable count per unit volume

N_t = viable count at time t per unit volume

D_{121} = D-value in minutes at 121°C

IF = inactivation factor

For the sterilization of aqueous preparations by heating in an autoclave, the British Pharmacopoeia (1988) considers satisfactory, a microbiologically-validated steam sterilization process that delivers, in total, an F_o-value of not less than 8 to every container in the load.

1.5 VALIDATION OF STERILIZATION PROCESSES

Sterilization processes are designed to destroy or eliminate viable life form from a product. Sterilization cannot be measured directly and indirect methods to assess the adequacy of the sterilization process are often used. During the design of a sterilization protocol, a reference organism is selected which should be more resistant than the most resistant of the likely contaminating organisms. Prior knowledge of the types, numbers and sensitivities of the contaminating organisms is necessary in these designs. Bacterial spores are often considered to be the most

resistant organisms to most lethal agents. Biological indicators (BI) are calibrated, resistant bacterial spores in a measurement system and have been shown that if properly calibrated and used, are effective tools for sterilization process validation and monitoring (12). Sterility tests are always recommended following any sterilization process but these are destructive on selected samples and results take time to show whether a batch can be passed or failed as sterile. There are also inherent problems of representative sampling and the task of proving that all units of a batch are sterile must involve the employment of probability statistics.

For proven methods of sterilization assurance to the efficacy of the process can be provided by monitoring the process by physical or chemical means. Measurement of temperature, time and pressure can be employed for the thermal processes, radiation dose and duration can be used for the ionising or U.V. radiation processes. Chemical indicators which integrate time and temperature by means of calibrated chemical reactions producing visible colour changes, are also available for monitoring some sterilization processes. For example, the Black spot Browne's Tube (Albert Browne Limited, Leicester) changes from red through amber to green after 15 minutes to indicate satisfactory exposure in an autoclave operating at 121°C. However, these chemical indicators are unreliable since they can change the colour to indicate sterility, but with a load that would fail a sterility test. Accurate temperature-time profiles are routinely used to validate heat

sterilization processes that have been established on the basis of the responses of marker organisms to the process and the acceptable probability of achieving sterility.

The use of toxic gases in sterilizing thermolabile materials involves the interaction of a number of parameters. In LTSF for example, there is potential for variation in temperature, pressure, formaldehyde concentration, homogeneity of environment, penetration of gas and humidity. It is not practical for the purpose of quality assurance to use physical and chemical methods to integrate all parameters. Biological monitoring is therefore essential but depends upon the availability of a suitable and reliable monitor.

1.6 BIOLOGICAL INDICATORS

1.6.1 Terminology

The definitions below were drafted for use in the British Standard Specification for Biological Indicators of Sterilization Processes (7).

1.6.1.1 Carrier

This is defined as the supporting material on which the spores are deposited.

1.6.1.2 Test Piece

This is the carrier on which a defined number and type of spores has been deposited.

1.6.1.3 Primary Pack

This is the container system for the test piece which has a defined permeability to the sterilizing agent(s) of the process in which it is to be used.

1.6.1.4 Biological Indicator or Biological Monitor

This is a test piece contained within its primary pack ready for use in the relevant sterilization process without further modification.

1.6.2 The Use of Biological Indicators

The first recorded use of Biological indicators in sterilization process validation was by Koch in 1881 (13). Biological indicators indicate sterility by inference provided they are used as a microbiological process control or to develop a sterilization cycle for a specific product. It would be convenient to have an ideal biological indicator that could be used for all situations but each product represents a unique bioburden. The design of each product sterilization process must be related to the

destruction characteristics of the chosen biological indicator as process lethalities depend on the product and on the types of the main contaminating organisms.

Currently the most frequently used biological indicators are prepared from spores of the family Bacillaceae. A number of biological indicators are routinely used to validate different sterilization processes during commissioning. Spores of B. stearothermophilus have been recommended for use as biological indicators for both moist and dry heat processes (4, 14). B. pumilus spores have been recommended for validating ionizing radiation sterilization (4, 15) and spores of B. subtilis var. niger are currently being used for ethylene oxide (EO) sterilization (4, 16, 17). Spores of C. tetani, atoxigenic, have been advocated for use as a suitable monitor for heat processes (18) but there are problems in cultivation. Cl. botulinum is reported to have been used in the food insutry as a reference organism for heat processes (2).

Although low temperature steam and formaldehyde sterilization processes (LTSF) are becoming widely accepted as alternatives to ethylene oxide sterilization (EO), there are no reports of biological indicators specifically designed for this process. The use (in LTSF sterilization) of biological indicators designed for moist heat processes has produced unreliable results (19, 20, 21) and B. subtilis var. niger, used in EO sterilization is sensitive to temperatures employed in LTSF sterilization. There

is therefore a need for a biological indicator to challenge and determine the efficacy of LTSF sterilization processes. As a consequence research projects are currently being conducted to develop a biological indicator for LTSF processes (22). Once such an indicator has been developed the failure of an LTSF cycle would be detected by the growth of bacterial spores after removal from the load into which they had been placed as a challenge to the process. The validity of the challenge relies on the ability of the LTSF-damaged spores to grow on a suitable recovery medium which will support the growth of the maximum number of survivors. The development of optimal recovery media is an area of current research.

During the design of sterilization processes, the level of treatment, e.g. temperature and time for heat processes, is based on the knowledge of the quantitative response of the chosen reference bacterial spores to the chosen treatments. The response of the spores is expressed quantitatively in terms of microbial death kinetics (Section 1.4).

1.7 DESIRABLE PROPERTIES OF BIOLOGICAL INDICATORS

Bacterial spores are used almost exclusively in Biological Indicators for the validation of many sterilization processes. This use allows for the efficiency of the sterilizing process to be directly assessed instead of being indirectly assessed by chemical or physical methods. Bacillus spores are commonly used as

biological Indicators due to their extremely high resistance to most inactivating agents. Spores to be used in the preparation of biological indicators must be easily grown, harvested and cleaned. Non-pathogenic strains are preferable to minimise introduction of pathogens into products to be sterilized. Aerobic strains are much simpler to grow than the anaerobic strains. Marker organisms should be easily identifiable to distinguish them from contaminants that might be introduced after the process. Thermophilic strains grow at higher temperatures than the optimal for most airborne contaminants and their use as biological indicators greaterly reduces the risk of false positive results. As high a Germination Index as possible is another prerequisite for spores to be used as biological indicators. The Germination Index (GI) is the ratio of the viable spore count to the total spore count of a spore suspension when expressed as a percentage. Spore populations of low Germination Indices often require activation before they can be inactivated due to the presence of dormant spores and this may lead to difficulties in distinguishing between dormant and inactivated spores. Furthermore, spores of a high Germination Index will enable statistical inferences based on the majority of the spore population rather than on the germinating minority in cases of low Germination Indices.

While the properties discussed so far are compromisable, the most important and uncompromisable property for a Biological Indicator is that of consistent and reproducible inactivation characteristics to the particular sterilant used. Ideally a

Biological indicator organism should exhibit Type A survivor curves to the inactivating process for accurate calculation of inactivation parameters. The most resistant spore strain to a specific sterilization process would be the best to use as a biological indicator since it offers a wider safety margin in practice. However, high resistance and linear inactivation kinetics are not necessary as long as the biological indicator organism has consistent and reproducible resistance characteristics. Biological indicator organism must also be stable in its characteristics upon storage both in suspension and when dried on a suitable carrier.

1.8 STRUCTURE OF THE BACTERIAL SPORE

Bacterial endospores are formed chiefly by members of the genera Bacillus and Clostridium. Spores may also be formed by Sporosarcinae and thermophilic Actinomycetes (23). In general, bacterial spores are highly refractile and often appear as phase bright structures when viewed under phase-contrast microscopy. They do not take up simple microbiological stains and are usually more resistant to lethal agents compared to vegetative cells. Mature spores have the ability to remain dormant for very long periods of time. Bacterial spores are rudimentally compartmentalised and rather complex in structure compared to vegetative cells (Fig. 3). The structure consists of a central protoplast or core which is the cryptic germ cell containing the cytoplasm, nucleoplasm and plasma membrane. The protoplast is unique in containing extraordinary concentrations of calcium and other minerals, dipicolinic acid

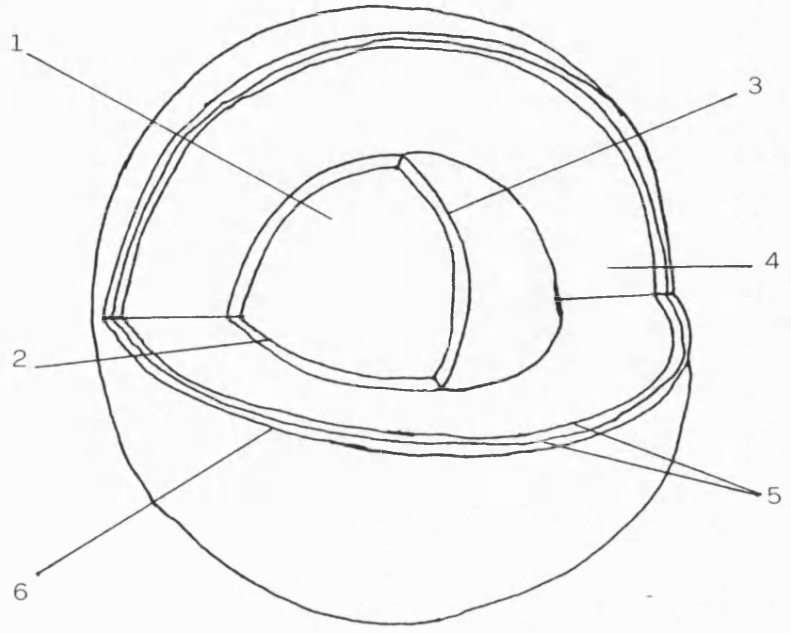


Figure 3. Diagrammatic representation of the main features in a bacterial spore.

From Gould, G.W. (24)

Key: 1 Protoplast (core)

2 Plasma membrane

3 Cortical membrane

4 Cortex

5 Spore coats

6 Exosporium

(DPA) (25) and small proteins which are all electrically and osmotically inactive (26). The protoplast is surrounded by a multilayered integument consisting of a cortical membrane, cortex, outer membrane, spore coats and exosporium. The cortex consists chiefly of a three dimensional matrix of electronegative peptidoglycan (27). The spore coats are proteinaceous in nature with small amounts of complex carbohydrate and lipid (28) and are resistant to attack by enzymes and some chemical agents (29). Spore coats have been demonstrated to protect spores from lysis and killing by hydrogen peroxide (30) and against killing by chlorine (31).

1.9 BACTERIAL SPORE RESISTANCE AND HOMEOSTASIS

Bacterial spores have been known to be resistant to heat, chemical agents and irradiation for many years. However the mechanisms by which spores achieve this resistance are still not established. A number of possible mechanisms have been proposed over the years with some still requiring the accomplishment of experimental verification.

Spores have the ability to remain dormant for long periods of time with reports of survival for 300 years in dried soil (32) and 1000 years for spores recovered from lake mud (33). The maintenance of a highly stable structure and physicochemical composition in adverse environmental conditions especially heat, is characteristic of dormant bacterial spores (26). Unlike vegetative

cells that tend to be stabilized by active processes, dormant spores are devoid of activity and unable to generate compensatory biochemical reactions against environmental change. The passive mechanisms responsible for maintaining the dormancy or refractory homeostasis in bacterial spores are often considered the same as those involved in the resistance shown by bacterial spores. Most of the experimental evidence bears on thermoresistance because it is of greater practical importance and can be readily quantified. It is well established that spores do not exhibit equal resistances to physical and chemical agents and also that spores of different species have markedly different resistances to chemicals and physical insults (34). The mechanism of spore resistance to heat may explain resistance to chemicals and other lethal agents but since there is usually no correlation between resistances to different agents, other factors must be involved (35).

Dormancy and resistance are thought to involve the stabilization of all vital, potentially labile, components within the spore. A number of different hypotheses have been postulated to distinguish between the physicochemical bases accounting for stabilization of essential structures and macromolecules, physiological mechanisms accounting for attainment of stabilization and mechanisms accounting for the maintenance of the stabilization (26). Biological indicators intended for validating and monitoring LTSF processes will be subjected to both heat and chemical insult and possible mechanisms of resistances to these agents will be reviewed.

1.9.1 Moist Heat Resistance

The unique and ubiquitous presence of calcium dipicolinate (CaDPA) in spore protoplasts led to the early speculation that it was a stabilizing factor for the labile essential spore components e.g. proteins (36). Dipicolinic acid has since been dismissed as part of the mechanism of spore thermoresistance since the isolation of DPA negative mutants which produced dipicolinic acid-free spores but were nevertheless still thermoresistant (37, 38). Furthermore, no correlation has been found to exist between DPA content and thermoresistance (39) and removal of the CaDPA complex and other low molecular weight spore substances had no effect on spore heat resistance (40). Low water content (or water activity) in the spore protoplast has long been associated with resistance. Currently spore thermoresistance is attributed to three main and measurable physicochemical factors that affect the vital protoplast. These are dehydration, mineralization and thermal adaptation (26). Dehydration of the protoplast is mainly responsible for the heat resistance of spores, with experimental evidence limited to the highly refractile core and non-refractory cortex.

Gerhardt et al. (41) demonstrated with dormant spores of B. stearothermophilus ATCC 7953, B. cereus T and B. subtilis var. niger that an exponential increase in moist heat resistance correlated with the refractive index of the entire spore and also showed that as the protoplast became more refractile and therefore more dehydrated, the moist heat resistances became greater among

the various spore types studied. Various attempts have been made to quantify water contents of protoplasts *in situ*. The use of genetically coat defective, bacterial spores made it possible for compartmental water content to be determined by equilibrium permeability measurements using ^{3}H-labelled water for entire spore, ^{14}C-labelled sugar for the cortex and by difference for the protoplast (42). In subsequent studies involving the use of buoyant wet density of protoplasts as a measure of water content (43) the extent of dehydration for different spores of *B. megaterium* was shown by Beaman *et al.* (42) to vary from 27-29%. This was considered to account for their relatively low heat resistance (42). In another study involving different spore species, the extents of dehydration varied between 30-55% and again showed good correlation with heat resistance. The resistances were much greater which suggested additional factors to protoplast dehydration as responsible for spore heat resistance (44).

Spore protoplasts contain extremely high concentrations of minerals mainly calcium and also iron, manganese and magnesium (34). Bender and Marquis (45) demonstrated that demineralization of spores decreased their heat resistance but that the resistance was regained on remineralization. Gerhardt (26) also reported that the extent of heat resistance is governed by the types and amounts of mineral ions in the spore. Mineralization is therefore considered the second factor to be implicated in the heat resistance of bacterial spores.

Thermal adaptation has long been known to affect spores intrinsically and is the third factor to be implicated in thermoresistance. Spores of thermophilic species are generally more resistant than those of mesophilic or psychrophilic species (46). Warth (46) demonstrated that spores of a given species grown at maximum temperature were more resistant than those grown at optimal or minimum temperatures.

In a study involving 28 different spores from seven Bacillus species spanning a wide range of resistance, the resistance was correlated with protoplast dehydration, mineralization and thermal adaptation. Beaman and Gerhardt (47) showed that increases in mineralization or thermal adaptation (or sporulation temperature) decreased protoplast water content by between 28% and 57%, which correlated with increases in spore resistance. Above and below these limits, spore resistance correlated with mineralization and thermal adaptation independently of protoplast water contents (Fig. 4). Of the three factors, dehydration is the predominating factor influencing bacterial spore resistance to inactivation by heat processes.

A number of explanations have been proposed to explain the attainment and maintenance of a dehydrated protoplast. One explanation is based on the hypothesis that the cortex exerts a pressure on the protoplast maintaining its dryness while the cortex and coats and exosporium, if present, are more hydrated. The cortex is thought to be able to maintain dryness and closepacking within

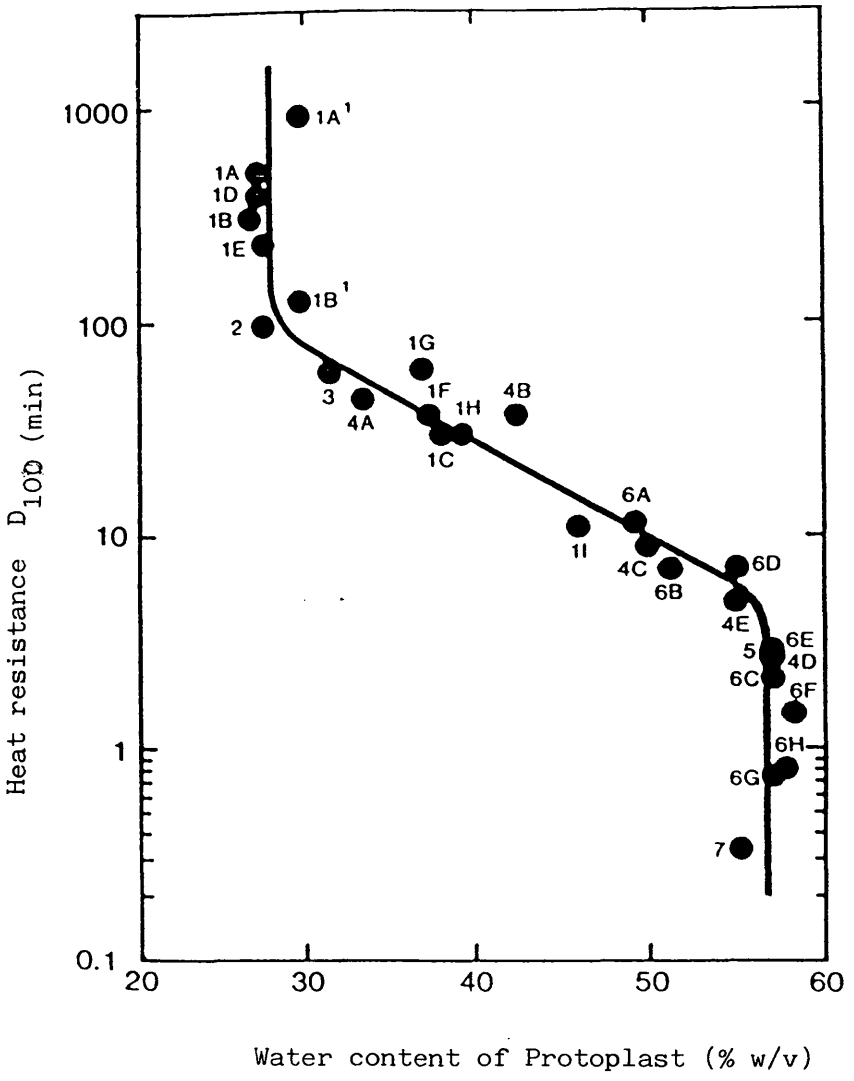


Fig. 4. Heat resistance correlated with protoplast water content of 28 spore types from 7 Bacillus species varying in thermal adaptation and in mineralization.

Key: 1A-1I B. stearothermophilus strains
2 B. caldolyticus
3 B. coagulans
4A-4E B. subtilis strains
5 B. thuringiensis
6A-6H B. cereus
7 B. macquariensis

From: Beaman and Gerhardt (47)

the protoplast apparently because of the compression of the peptidoglycan network. Spore peptidoglycan has lower degrees of cross linking with many muramyl residues and few substituent peptides. This results in a net negatively charged loose elastic structure (28). The relative volume of cortex peptidoglycan in spores is much larger than in vegetative cells and the relative volume of cortex correlates with spore heat resistance (48). It was proposed by Warth (28, 40) that the cortex in the intact spore was in a contracted state with tension directed radially inwards to the protoplast.

Molecular mechanisms in the cortex for the attainment and maintenance of a dehydrated protoplast have also been proposed. The expanded cortex hypothesis of Gould and Dring (49) proposed an osmoregulatory model in which the cortex exerted a swelling pressure inward on the protoplast and outward against the coat. The hypothesis for this model has been disputed and instead, a case for a swelling pressure of an anisotropic cortex exerted radially inward against the protoplast proposed (28). There has also been an argument for the case for contraction of the cortical peptidoglycan in terms of the acid/base physiology and the unusual properties of cortical peptidoglycans thereby exerting a pressure on the protoplast (26, 45). However, there are no experiemntal results to verify the argument.

1.9.2 Chemical Resistance

Bacterial spores show different resistances to chemicals, irradiation and heat. While the mechanisms responsible for attaining and maintaining an intact spore structure may be as significant in chemical resistance as in heat resistance, the spore coat and cortex have extra roles in chemical resistance. There is enough evidence now that sporulating cells become progressively resistant to non-antibiotic antimicrobial agents (34). In studies using sporulating B. subtilis, resistance was progressively attained first to octanol, followed by butanol with resistance to chloroform, methanol, ethanol, phenol appearing at the same time immediately after (50, 51). When these events were correlated with the maturation of the cortex and deposition of coat material in the sporulation cycle, resistance to the chemicals was observed to occur before the stage when spores acquired heat resistance (34, 50, 51). Resistance to bactericides such as povidone-iodine, glutaraldehyde and hypochlorite coincided with the coat formation stage of sporulation (52).

The spore coat has been reported to protect against the effects of ethylene oxide (53), hypochlorous acid (52, 54) and octanol (50, 51, 55). The sporicidal activity of Betadine at 37°C on normal and coat defective spores showed an increasing rate of kill with increasing severity of coat extraction (56). The coat offers its protection against some chemicals by impermeability due to its disulphide-rich protein and alkali-soluble protein (34).

Disruption of these disulphide bonds in spores of Cl. bifermentans, B. cereus and B. subtilis, reduced their resistance to hypochlorous acid (31) and was also reported by Gould and King (57) to increase spore sensitivity to hydrogen peroxide.

Although the coat provides protection from some chemicals, it still remains permeable to most chemicals (34). An intact spore coat provided no protection from glutaraldehyde in B. pumilus species (58). It has been reported that spore coats may be protective in one species but not in another (54). Coats form an effective barrier against hydrogen peroxide in Cl. bifermentans but not in B. cereus species (34).

The protoplast has also been implicated in chemical resistance of spores. Spores of Cl. bifermentans have protoplasts with a high affinity to bind copper (II) ions and lead (II) ions (59). Waites et al. (60) demonstrated that pre-incubation with copper (II) ions reduced the resistance of these spores to hydrogen peroxide but had no effect on resistance to hydrogen peroxide of spores of Cl. sporogenes PA 3679, Cl. perfringens NCTC 8238, B. subtilis var. niger and B. cereus. These authors also showed that although the rate and extent of the uptake of copper (II) ions by all the spores studied was similar, copper (II) ions were only bound by the protoplasts of spores of Cl. bifermentans (60). These spores of Cl. bifermentans were also less resistant to a combination of glutaraldehyde and formaldehyde when compared to the non-binding species (35).

1.10 FACTORS AFFECTING SPORE RESISTANCE

Since bacterial spores to be used as biological indicators must be of consistent and reproducible resistance to the sterilizing processes, factors affecting their resistances, especially during production and treatment must be considered carefully.

The factors affecting bacterial spore resistance can be divided into three categories as

(i) Genetic and intrinsic factors

(ii) Influences during growth and sporulation

(iii) Influences during treatment and recovery.

The latter two can be considered extrinsic (61);

1.10.1 Intrinsic Factors

The resistance of bacterial spores to heat, chemicals and radiation is characteristic of the genotypes and therefore varies between spores of different species and strains. The genetic variation in resistance between different strains of the same species can be of a considerable magnitude (62). Two strains of *B. cereus* were reported by Bradshaw *et al.* (63) to have D_{121}-values that varied by a factor of 78 and Roberts (64) reported seven strains of *Cl. welchii* that varied in their D_{90}-values by a factor of 48. Generally thermophilic strains are more resistant than spores from mesophiles and psychrophiles. It has been reported by

many authors that in the same medium and under the same growth conditions, different strains of the same species can produce spore suspensions having widely different resistance. This is illustrative of inherent resistance (20). Given the same genotype, some organisms such as B. stearothermophilus show considerable phenotype variations and this could explain variations in resistance observed within members of the same species.

1.10.2 Influences During Growth and Sporulation

Influences on spore resistance active during growth and formation of spores can be illustrated by the effects of incubation temperature and composition of sporulation medium (20, 65)

1.10.2.1 Composition of Sporulation Medium

There is a lot of published and often conflicting literature on the effects of various components in the sporulation medium, on the resistance of bacterial spores. Different species and strains have different nutrient requirements and variation in quality and quantity of nutrients will result in spores of different structure and of varying resistance. Generally divalent cations in the medium improve thermal resistance of bacterial spores (66). Calcium is thought to protect spore enzymes from inactivation by forming complexes with dipicolinic acid. The DPA/cation ratio was considered critical and important in infering heat resistance to spores (61). The inclusion of manganese ions in

the sporulation medium, was reported to increase thermal resistance of spores of B. fastidious (67) and of B. megatesium ATCC 8245 (68). Molin et al. (69) reported that the proportion between glucose, sucrose and calcium in the sporulation medium strongly affects the dry heat resistance of B. subtilis spores. Addition of sucrose to a calcium-rich medium improved both sporulation and dry heat resistance. In calcium deficient medium, the effect of sucrose was reversed (69). Cook et al. (70) reported that sodium chloride weakened the thermal resistance of spores with the effect increasing with storage time. Hoxey (22) demonstrated the variation of spore resistance to inactivation by moist heat and aqueous formaldehyde solution with different chemically defined sporulation media. Because complex media often produce spore suspensions of the same strain but with inconsistent resistance, chemically defined media have always been recommended for the production of spores of consistent resistance, for example spores for use as biological indicators. Phosphate concentration has often been cited as important in conferring thermal resistance in spores but reports are often contradictory. High phosphate levels have been reported to decrease resistance (71, 72) but a sporulation medium containing 0.05% phosphate was demonstrated to produce spores that were more resistant than those from a medium from which phosphate had been omitted (73).

1.10.2.2 Incubation Temperature

Increasing sporulation temperature has been known to

increase thermal resistance (46). Warth (46) demonstrated that for aerobic thermophiles, spores grown at maximum temperature were more thermo-resistant than those grown at optimal or minimal temperatures. For mesophillic, anaerobic spore formers, it appears that the lowest temperature consistent with good growth usually produces the most resistant spores (74).

In some cells, sporulation temperature seems not to have any effect on spore resistance. Dadd et al. (75) reported no effect of temperature on the resistance of B. subtilis spores to ethylene oxide. Similarly, thermal resistance of Cl. perfringens strains was not modified by sporulation temperature (76). Increase in sporulation temperature is suspected of increasing the DPA/cation ratio in spores which could be of some significance in heat resistance (34).

1.10.2.3 pH

There seems to be no correlation between the pH of sporulation medium and thermal resistance of spores (77). The pH of unbuffered medium will gradually fall during the growth of cells. Different glucose concentrations were reported to produce spores of Cl. sporogenes of differing thermal resistance (78) but on keeping the pH constant at 7, the variation in glucose concentration had no effect on the thermal resistance of the spores (78). Buffering agents were included in the compositions of the chemically defined media used throughout this work to maintain a neutral pH.

1.10.3 Storage Effects

Data published on the effects of storage of spore suspensions on chemical and heat resistance are scarce and often contradictory. Cook and Brown (79) and Reich et al. (80) reported loss of moist heat resistance on storage at room temperature of B. stearothermophilus spores but Cook and Gilbert (81) reported increased moist heat resistance during storage of spores of the same species. No effect was reported on the resistance of B. subtilis spores to ethylene oxide after storage for periods up to 24 months (82). Leaper et al. (83) reported increased resistance of spores of B. subtilis var. globigii to peracetic acid following storage at 4°C in aqueous suspension but observed no change on resistance of B. subtilis SA22 spores to hydrogen peroxide after storage at 4°C for periods up to 134 weeks. Different workers store spore preparations in different environments and often use different methods to determine spore resistance to lethal treatment. It is therefore difficult to correlate results from different authors to a general conclusion.

The age of spore preparations has been associated with the degree of dryness or the moisture content (a_w) of the protoplast (84). Drying spores to known moisture contents was reported to increase the spore resistance to moist heat (85). Dried spores on carriers were reported to have a higher resistance to dry heat (86) and to ethylene oxide (87) than spores not dried to the same extent. Newly formed spores and very old spores tend to show lower

thermal resistance than the average mature spore (84).

1.10.4 Influences During Cleaning

All methods used in cleaning spore crops have been reported as having some effect on the resistance of spores to heat and chemical insult (35). Washing spore crops tends to lower spore resistance possibly due to removal of organic or inorganic materials which could be protective. Excessive washing has also been reported by Gorman et al. (52) as interfering with the spore coat which might play an important role in spore resistance to chemicals. Germinants and debris from sporulation media can also influence spore resistance (88). The method used to clean spore suspensions used in the work described later was free of any other chemical but sterile distilled water, to minimise interference with the spore resistances. The use of lysozyme in cleaning bacterial spore suspensions can modify spore resistance to heat and chemicals. Gould (89) reported that the use of lysozyme can increase the permeability of the spore coats thus sensitising the spores to some chemicals. Peptidoglycan in the outer part of the cortex is lysozyme-sensitive (89). Therefore the use of lysozyme could interfere with the mechanisms maintaining the dehydrated protoplast and thus modify spore heat resistance.

Sanz et al. (90) reported a marked decrease in the moist heat resistance of Bacillus stearothermophilus spores after ultrasonication treatment. The exact mechanism by which

ultrasonication treatment can reduce spore heat resistance is unknown but could be due to some changes in the spore structure. Ultrasonication was reported to remove exosporangia in spores of some strains of B. subtilis (89). Sonication methods are frequently used to remove sporangial material and to break up spore aggregates. Pre-treatment sonication should not be used in spores whose resistance is to be determined but post treatment sonication, e.g. in recovering spores off carriers does not seem to influence recovery (90). Heating spore suspensions at 80°C to kill any vegetative cells present was reported to reduce spore resistance to ethylene oxide treatment possibly due to changes in spore coat permeability (75).

1.10.5 Influences During Treatment and Recovery

1.10.5.1 Presentation of Spores to Sterilization Treatment

The manner in which spores are presented to the test conditions can have considerable influence on the resistance of spores to the lethal agent. In moist heat resistance studies, spores are often presented in a suspending medium. In gaseous resistance studies, spores are often presented to the conditions dried on suitable carriers. In both these situations, there is potential for interactions between the suspending or carrier medium and the organism, increasing or reducing its sensitivity. The physico-chemical nature of the carrier material could also influence spore resistance. The moisture content in spores dried on

carriers is of importance since it has significant effect on chemical resistance (87). Constituents of suspending medium could also interfere with the lethal agent under test by potentiating or retarding its lethality (91, 92). Studies of the influence of suspending medium on spore thermal resistance have been extensive (34, 93). Buffers used in controlling pH of suspending medium have been reported to influence spore resistance (94, 95). The presence of 6% w/v sodium chloride has been reported to have no effect on thermal resistance of aerobic spores but concentrations of 0.5-1% w/v caused an increase in resistance while in the presence of 8, 10 or 20% w/v, the resistance was reduced (96). Peptone water was reported as offering slight protection to spores exposed to heat inactivation (97). Buhlmann et al. (98) observed a much higher heat resistance of Bacillus stearothermophilus spores suspended in physiological saline than those suspended in water.

Reports on the effects of pH of suspending medium on spore resistance are conflicting. Usually spores are most stable at neutral pH with variation of pH between 5 and 9 having minimal effect (93, 96). B. stearothermophilus spores showed a higher resistance at pH of 7.2 than at pH of 6.5 (99). Cl. tetani spores have been reported as more sensitive at acid pH than alkaline pH, while in contrast, Cl. perfringens and Cl. sporogenese spores were reported to be more thermosensitive at alkaline pH than at acid pH (34). Gould (89) reasoned that low pH values could result in the protonation of some structural components of the spore, for example the cortex peptidoglycan to reduce its contractile or expansive

pressure exerting functions on the protoplast which would allow the protoplast to partly rehydrate and thus lose its resistance to heat. High alkali pH is thought to remove alkali-soluble coat proteins and increase permeability which could influence both moist heat and chemical resistance in spores (89).

In general, since the medium in which spores are treated has considerable effects on thermal and chemical resistance, resistance tests on spores to be used for validation of sterilization processes should preferably be performed in a medium similar to the materials that are going to be subjected to the process. It is also difficult to compare heat resistance or chemical resistance data from different workers since they have invariably used different spore production and test procedures.

1.10.5.2 Influences During Recovery

Microbial death has often been defined as the inability of an inactivated organism to reproduce in a suitable environment, that can be demonstrated to enable reproduction of untreated control organisms (34). In this context, recovery is the ability of a spore to germinate and grow to produce visible colonies on solid medium or turbidity in liquid medium and differs from revival or repair (34). An incorrect choice of recovery conditions can lead to an erroneous assessment of spore resistance. It is therefore important to provide optimum conditions for germination, outgrowth and multiplication of survivors to have an accurate determination

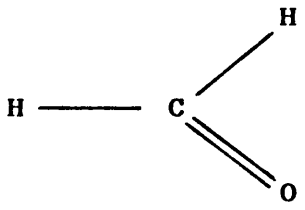
of resistance. The composition of recovery medium can influence recovery. Enriched media have often been used to enhance recovery of damaged spores since these are often more precise in their growth requirements than untreated spores. Sometimes growth factors have been included in the recovery media to enhance recovery (61). Some substances present in the recovery media can inhibit or enhance germination and outgrowth of treated spores. In studies where spores are exposed to chemicals, it is always important to inactivate the lethal agent at the sampling time. Some of the inactivators that have been used have been shown to inhibit germination and growth. Long chain fatty acids in the recovery medium have been shown to inhibit germination (61) and cell division as demonstrated in Cl. botulinum (100). The addition of starch has been reported to neutralise the effect of the fatty acid (101). Thioglycollate broth is inhibitory to the outgrowth of Bacillus species and various Clostridium species (102, 103). Other recovery media constituents that have been reported as inhibitory to recovery include bromocresol purple (present in Oxoid Dextrose Tryptone Broth) (104) and bromothymol blue (98) to heat treated spores of B. stearothermophilus.

The pH of the recovery medium also affects recovery. The optimum pH for recovery of B. stearothermophilus spores is considered to be pH 7.4 (105, 106). The optimum pH for recovery of B. subtilis spores exposed to ethylene oxide is reported as 6.9 (75).

Incubation temperature and the period of incubation can also affect recovery. Generally, the optimal recovery temperature for heated spores is below the optimum growth temperature of untreated spores (81, 107). Cook and Gilbert (81) reported an optimum recovery temperature of heated spores of B. stearothermophilus at 45-50°C, which is below the optimum growth temperature of unheated B. stearothermophilus spores of 56-60°C. Prentice and Clegg (107) reported an optimum recovery temperature of heated spores of B. subtilis at 30°C, lower than the optimum growth temperature of untreated spores at temperatures between 16-48°C. These authors (107) concluded that outgrowth rather than germination was sensitive to temperature.

Treated spores might need longer time periods for germination and growth than untreated spores and therefore, the incubation time before assessing survival during resistance tests must be long enough to ensure optimum recovery.

1.11 PHYSICO-CHEMICAL PROPERTIES OF FORMALDEHYDE



Mwt = 30.2

Mp = -118°C

Bp = -19°C

From Walker (109)

Synonyms: Methanal, formic aldehyde, methyl aldehyde.

Pure dry formaldehyde gas is colourless characterised by a pungent

odour and is irritant to mucous membranes of the eyes, nose and throat at concentrations above 20 ppm. Both the gas and liquid polymerise readily at room and lower temperatures to produce a white film of polymer on container walls. There is no visible polymerisation at temperatures in excess of 80°C for the pure dry gas which obeys the ideal gas laws without pronounced deviation (108). Impurities such as traces of water, initiate rapid polymerisation at room temperature to polyoxymethylene glycol. Paraformaldehyde is a mixture of polyoxymethylene glycols contaiing 90-99% formaldehyde and a balance of combined and free water. The gas also polymerises to a trimer, trioxane, which is a cyclic polymer. Trioxane is non-irritant to mucous embranes and possesses a pleasant odour. These polymeric forms gradually vaporize generating monomeric formaldehyde gas at a rate dependent on temperature.

1.11.1 **Formaldehyde in Solution** (synonym formalin)

Anhydrous formaldehyde gas is moderately soluble in non-polar solvents like chloroform and toluene and readily soluble in polar solvents. Anhydrous formaldehyde dissolves in water exothermically (109). Dissolved formaldehyde is present principally as the monohydrate, methylene glycol $CH_2(OH)_2$ together with a series of low molecular weight polymeric hydrates. Low concentrations favour methylene glycol but the equilibrium shifts towards higher molecular weight polyoxymethylene glycols as the concentration increases. Commercial formaldehyde solutions

(formalin) contain 37-40% w/v formaldehyde with 10-15% methanol as stabilizer to prevent precipitation of polymers. Chemical reactions which may be significant in the bactericidal action of formaldehyde are the condensation or addition reactions (109). Formaldehyde will act as a nucleophile to undergo nucleophillic addition reaction with water and amines. This is the manner in which formaldehyde is considered to react with amino acids and the pyrimidine and purine bases in nucleic acids (110).

1.11.2 Review of Formaldehyde Inactivators

In any investigation of the bactericidal or sporicidal activity of an agent, it is necessary to ensure that the lethal action of the agent is terminated immediately at the sampling time to allow for accurate evaluation of the agent's activity. In moist heat resistance studies, the lethal activity is often terminated by rapid cooling of the samples in crushed ice to reduce the temperature to non-inactivating levels. Germination and outgrowth of surviving organisms should be unhindered to enable accurate determination of surviving organisms. The use of inactivators for investigations involving a chemical agent such as formaldehyde, will guarantee the termination of the formaldehyde induced lethality in bacterial spores and will avoid the carryover of bacteriostatic concentrations of the agent to the recovery medium. The use of inactivators has been demonstrated to produce greater reproducibility of results and leads to a more quantitative evaluation of antimicrobial agents under investigation (111).

Cheung and Brown (111) demonstrated that glycine, at 2% w/v concentration, could effectively inactivate 2% alkaline glutaraldehyde. Glutaraldehyde is an aldehyde just like formaldehyde and glycine has also been used to successfully inactivate formaldehyde (112). Other methods that have been used to inactivate formaldehyde include morpholine and dimedone (Nash, 112), sodium sulphite (Nordgren, 113)) and ammonia (Sprague, 114). Physical methods have also been used to remove formaldehyde from the samples and these include filtration (Hayes, 115) and centrifugation with washing (Spicher, 116). Suitable inactivating agents have also been included in the recovery medium (Russell, 117). In a comparative evaluation of inactivation potential of the above chemicals, Hoxey (22) demonstrated 10% w/v glycine to effectively inactivate concentrations of formaldehyde up to 35% w/v. Hurrell (74) has reported on the use of a 1% w/v glycine solution in inactivating 0.5% w/v aqueous formaldehyde solution and further reported its superiority to the use of the other related amino acids like cysteine.

1.12 HISTORY OF FORMALDEHYDE AS A BACTERICIDE AND THE DEVELOPMENT OF THE LTSF PROCESS

1.12.1 History

Formaldehyde has long been established as an effective bactericidal and virucidal agent with its bactericidal activities reported as early as 1886 by Loew (118). Since then it has been

used as a fumigant to disinfect large rooms as described by Aronson in 1897 (119). In 1899, Sprague (114) described a system for disinfection when he used a heated vacuum chamber. He demonstrated inactivation of B. anthracis spores by gaseous formaldehyde in a jacketed chamber heated to 90°C. In 1902, Esmarch (120) demonstrated the sporicidal action of gaseous formaldehyde when added to a subatmospheric pressure steam at 70°C on spores of B. anthracis. Nordgren, in 1939 extensively reviewed the history of formaldehyde and also studied the sporicidal activity of gaseous formaldehyde (113). He found that gaseous formaldehyde was more effective in an atmosphere of low humidity (up to 50% RH) and that activity increased with temperature and partial pressure of formaldehyde. In his conclusions, he stated that although formaldehyde was a powerful bactericide, it had only a limited capacity to sterilize unless elevated temperatures were used. The Public Health Service Committee on formaldehyde disinfection set up in 1958 confirmed Nordgren's findings and recommended the use of formaldehyde vapour as a disinfectant only if no alternative method was available (121).

Aqueous solutions of formaldehyde were demonstrated to be bactericidal against Staphylococcus aureus, E. coli and E. berthella at concentrations between 0.166% and 4% (122). Sporostatic and sporicidal activities of these aqueous solutions were demonstrated by Trujillo and David (123) on spores of B. subtilis var. niger. These authors (123) concluded that the sporostatic effect was a result of reversible inhibition of spore

germination whilst the sporicidal effect was due to the temperature dependent inactivation of these spores in aqueous formaldehyde.

1.12.2 Development of LTSF

In 1961, Alder and Gillespie (124) successfully disinfected woollen blankets using steam at subatmospheric pressure at temperatures below boiling point, in a suitably adapted autoclave chamber. Five years later, Alder et al. (125), working at Bristol Royal Infirmary, demonstrated sporicidal action of the procedure described in 1961 after the addition of formaldehyde vapour to the steam. This marked the first use of low temperature steam (LTS) with formaldehyde on a practical basis to sterilize heat labile hospital materials. The system described in 1966 involved a dry evacuation of air from an autoclave chamber followed by an injection of heated formaldehyde vapour and then sub-atmosheric steam (LTS). In this study, failures to sterilize were associated with failures of formaldehyde to penetrate layers of fabric and narrow bore-tubing like ureteric and cardiac catheters. The reasons were considered to be due to moisture condensation. Alder (126) in 1968, and two years later working with Mitchell (127), improved on the original design of 1966. They used a dry evacuation of air system but modified it by injecting and evacuating small amounts of formalin vapour followed by LTS in a superheated environment using unsaturated steam. Since then, the system has been adapted in other centres and has been found to be a useful and acceptable method of processing a wide variety of objects and materials. Various

researchers have since been contributing ideas about the system. Weymes (128) showed that the addition of gaseous formaldehyde to a humidified and evacuated chamber, followed by LTS, was sporicidal and assumed that the latent heat of LTS and the formaldehyde monomers had a synergistic and sporicidal effect. In 1980, Hurrell (129) proposed that the principal requirement for sterilization was a homogenous mixture of monomeric formaldehyde gas and saturated steam. Gibson (130) and Marcos and Wiseman (131) also emphasised the importance of a low humidity in their studies of the system. Line and Cutts (132) described a process in which controlled steam injection, enough to raise chamber pressure to 80-100 mbar, and introduced at an early stage before formaldehyde,was effective. In 1984, Hoxey (22) demonstrated sporicidal activity on a number of different bacterial spore strains usig a test apparatus based on a glass manifold system. Steam and air pulses were used to evacuate the chambers and to bring them to operating temperatures between 65° and 80°C, followed by admission of the formaldehyde vapour to a thermostatically controlled environment. The use of formaldehyde with sub-atmospheric steam (low temperature steam) has become widely known as Low Temperature Steam with Formaldehyde (LTSF) sterilization.

Differences of opinion have been voiced on relative roles of LTS and formaldehyde and also on how and when these chemicals should be applied. In 1987, Alder (133) described a modification of the original developments of 1966 and 1968 (125, 126) by using 'dry' formaldehyde gas initially and unsaturated water vapour at

later stages in the cycle for the sterilization procedure. Saturated water vapour was then used to elute residual formaldehyde at the end of the process. To distinguish the procedure from earlier developments, the recent modification has been called Formaldehyde with Low Temperature Steam (F/LTS).

1.13 PRINCIPLES OF LTS, LTSF AND FLTS

Low temperature steam (at subatmospheric pressure) LTS, is currently being used in hospitals to disinfect heat sensitive materials. The method is based on the principle that when saturated steam is admitted to a previously evacuated chamber, the steam temperature can be accurately maintained by controlling the chamber pressure (124, 134). Figure 5 shows the relationship between temperature of saturated steam and subatmospheric pressure.

Saturated subatmospheric pressure steam has more disinfecting energy than water at the same temperature. In the chamber, steam condenses and gives up latent heat energy while the sensible heat of the material stays at the set temperature. LTS is therefore capable of destroying non-sporing microorganisms at temperatures below that of boiling water (124, 134). Weymes (135) proposed that Low Temperature Steam with Formaldehyde utilises the synergistic antimicrobial effect of latent heat of LTS and the formaldehyde monomer.

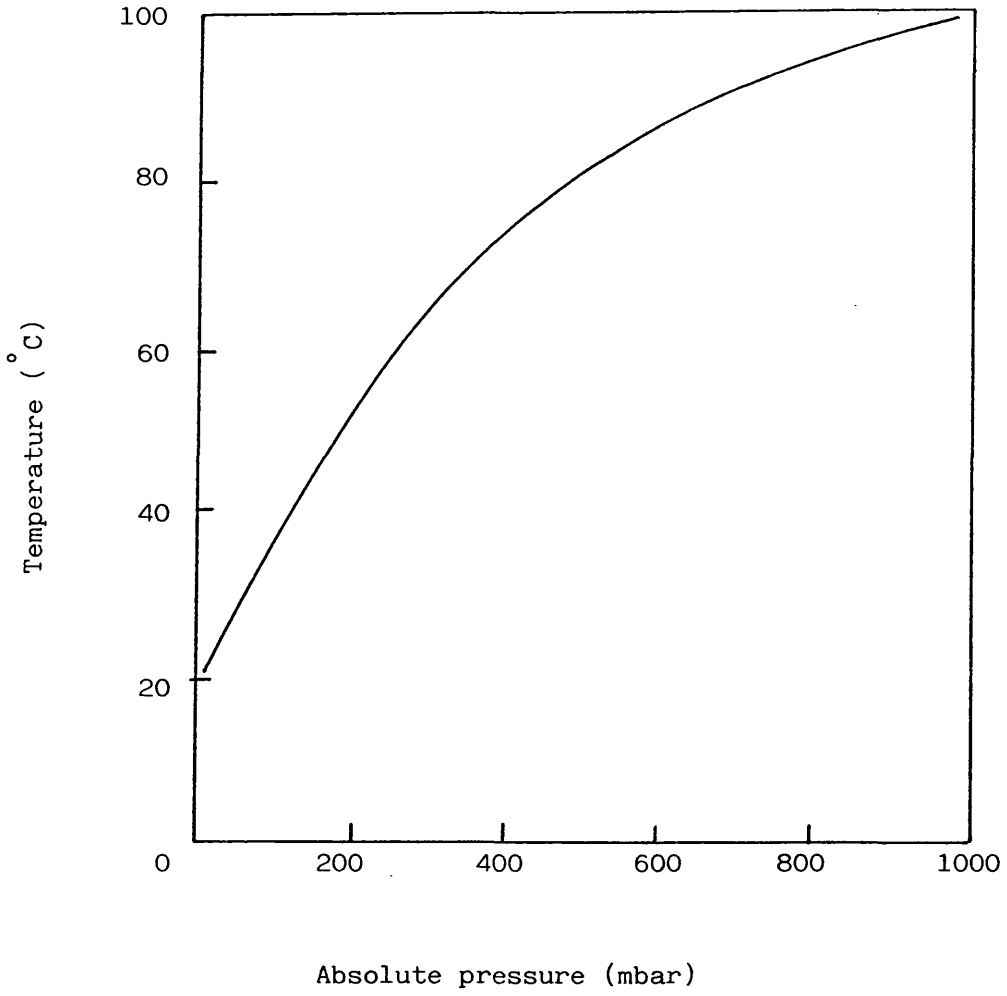


Fig. 5. The temperature of subatmospheric oressure steam.

From Alder and Simpson (134)

1.13.1 Operating Cycles

The latest developments of Alder (133) have been termed the Formaldehyde with Low Temperature Steam process (FLTS). The author demonstrated that the sporicidal activity on 'Oxoid' B. stearothermophilus spore strips started during the initial formalizing stage of the cycle when formaldehyde was adsorbed on the bacterial spore surfaces. The final addition of formaldehyde gas (generated from 2.96×10^2 ml of 38% w/v aqueous formaldehyde solution per litre of chamber volume) and LTS to raise the temperature to the required operating temperature (73°C), only complemented the sterilizing activity of the adsorbed formaldehyde. Figure 6 illustrates the pressure variation during a typical FLTS cycle.

SUMMARY OF STAGES OF FLTS

1. Dry evacuation of the chamber. The jacket temperature is set to 1.5°C above operating temperature to prevent moisture condensation and superheated environment.

2. Formaldehyde vapour from the formaldehyde vaporiser is slowly introduced in 3 separate injections at 3 minute intervals. Each litre of chamber space requires 74 ml of 38% w/v formaldehyde to be vaporized. This is the stage when formaldehyde gas, assisted by vacuum, penetrates narrow tubing and porous materials and is adsorbed onto proteinaceous surfaces.

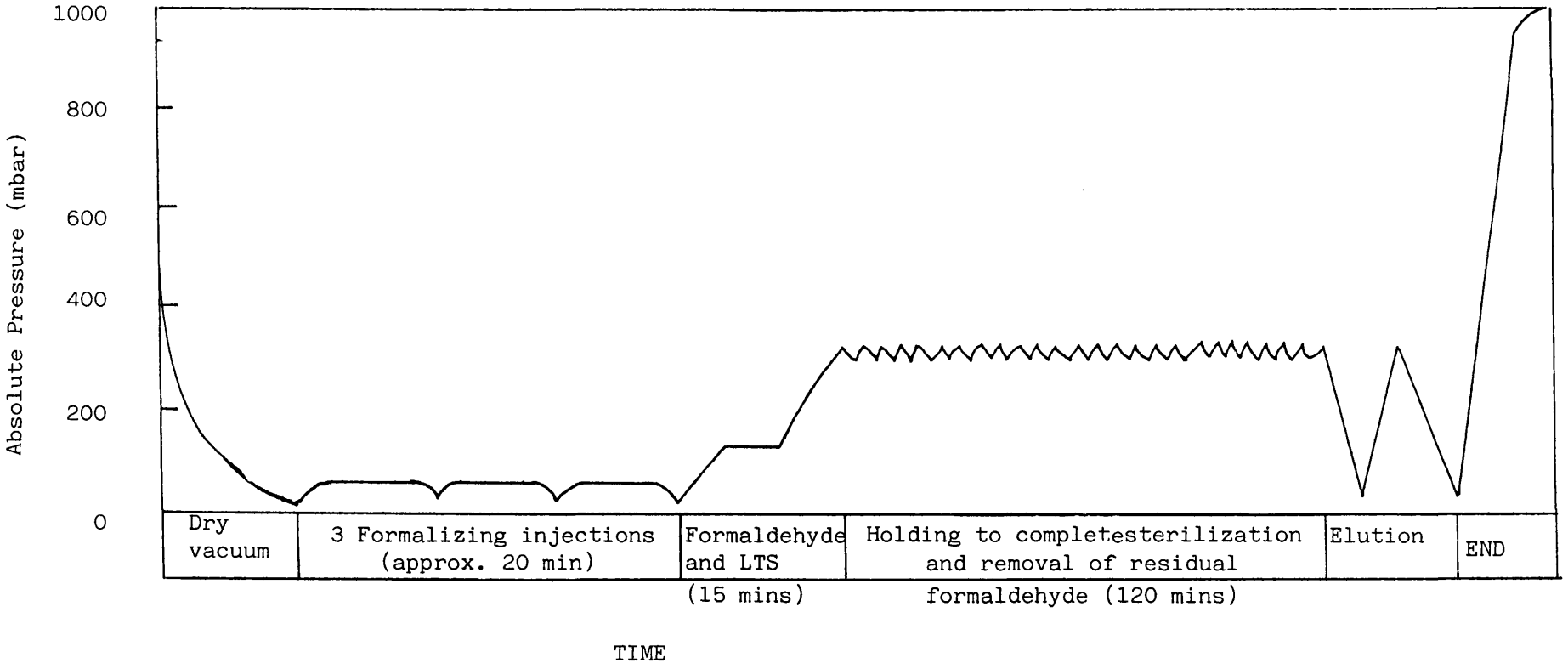


Fig. 6. Operating cycle of a FLTS sterilizer from Alder (133).

3. Formaldehyde vapour from the formaldehyde vapour 1.96 x 10^2 ml per litre chamber space) is admitted into the chamber until the pressure within the chamber has stabilized. After 3 minutes LTS is slowly introduced to the required temperature and pressure.

4. Steam is continually injected at the set temperature for 120 minutes to complete the sterilization stage and to remove residual formaldehyde through the chamber drain.

5. Evacuation stage. Further residual formaldehyde is removed by alternating steam pulses and vacuum for at least two pulses.

6. Aeration. Filtered air is introduced into the chamber to bring it to atmospheric pressure.

In this cycle, sterilization was indicated to have been completed early in stage 4.

Another variation in the use of LTS with formaldehyde was that of Weymes (136). The cycle consisted of an initial evacuation of the chamber to below 50 mm Hg followed by a series of 15 deep pulses of steam and formaldehyde between 50 mm Hg and 200 mm Hg at 65°C (37 ml formaldehyde solution for each litre of chamber space). The selected temperature and pressure were then maintained for a set period, usually 90 minutes. This was followed by five steam

pulses and finally elution of residual formaldehyde by three vacuum/air pulses between 50 mm Hg and atmospheric pressure. This cycle was developed for porous loads operating at much lower temperatures than those reported by Alder et al. (125, 133). Hurrell et al. (137) also described a multi-pulse system when the formalizing period was increased to 20 pulses and the hold period reduced to only 15 minutes. Steam was then flushed through the load to remove residual formaldehyde followed by air rinsing and degassing (Fig. 7).

Commercial LTS and formaldehyde sterilizers currently available do not conform to either of the cycle characteristics described above. Current cycles are automated consisting of an initial air removal stage utilising steam and vacuum pulses to bring the chamber to operating temperature and sturation pressure. At the set temperature and pressure, formaldehyde vapour from the vaporizer is admitted (49.14 mg l^{-1} chamber space) followed by steam. Temperature and pressure are then maintained for the sterilizing period of between 1-2 hours by alternating steam and vacuum pulses. At the end of the sterilizing period, residual formaldehyde is eluted by a series of steam pulses followed by a period of aeration to bring the chamber to atmospheric pressure. Figure 8 illustrates the pressure variation in a typical commercial LTSF cycle operating at 80° ± 2°C.

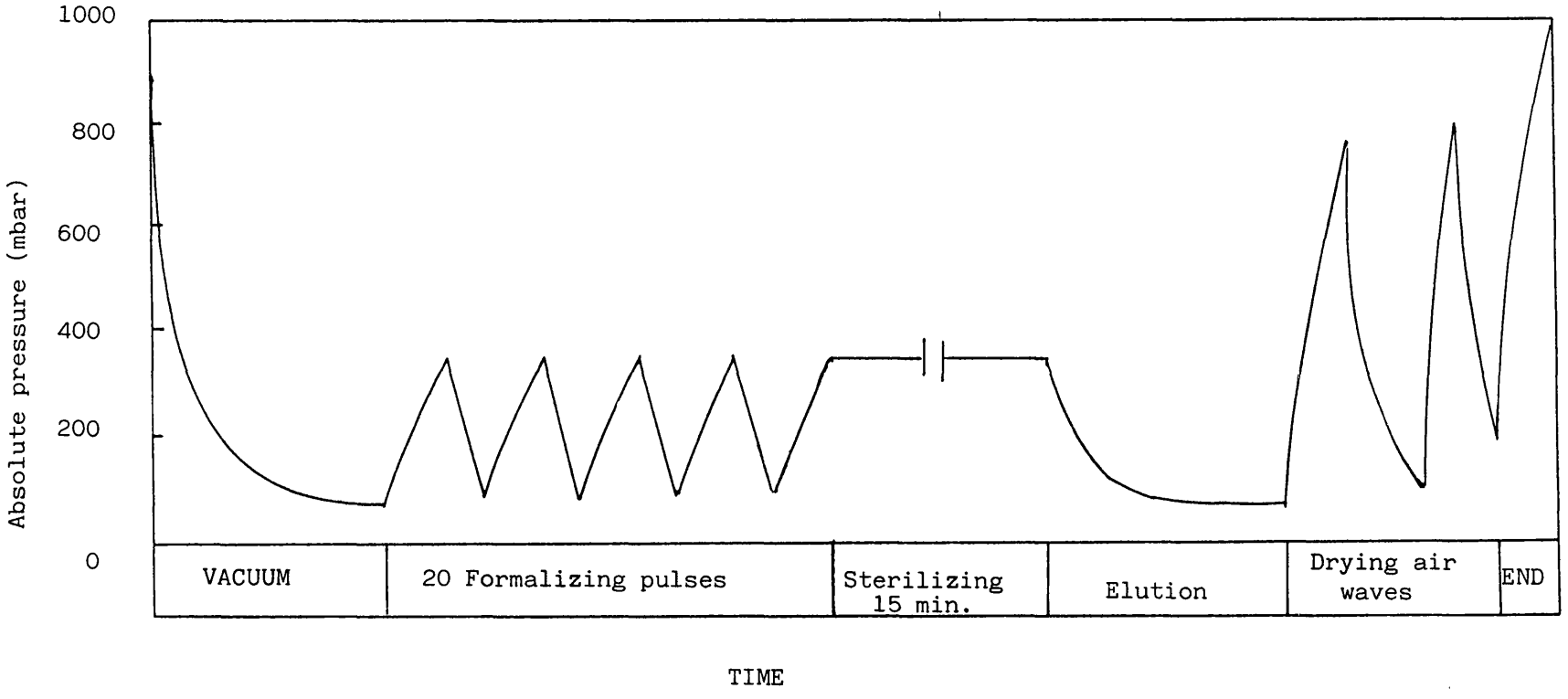


Fig. 7. Representation of a multiple pulse LTSF cycle at 73°C[3] From (Hurrel et al. (137)

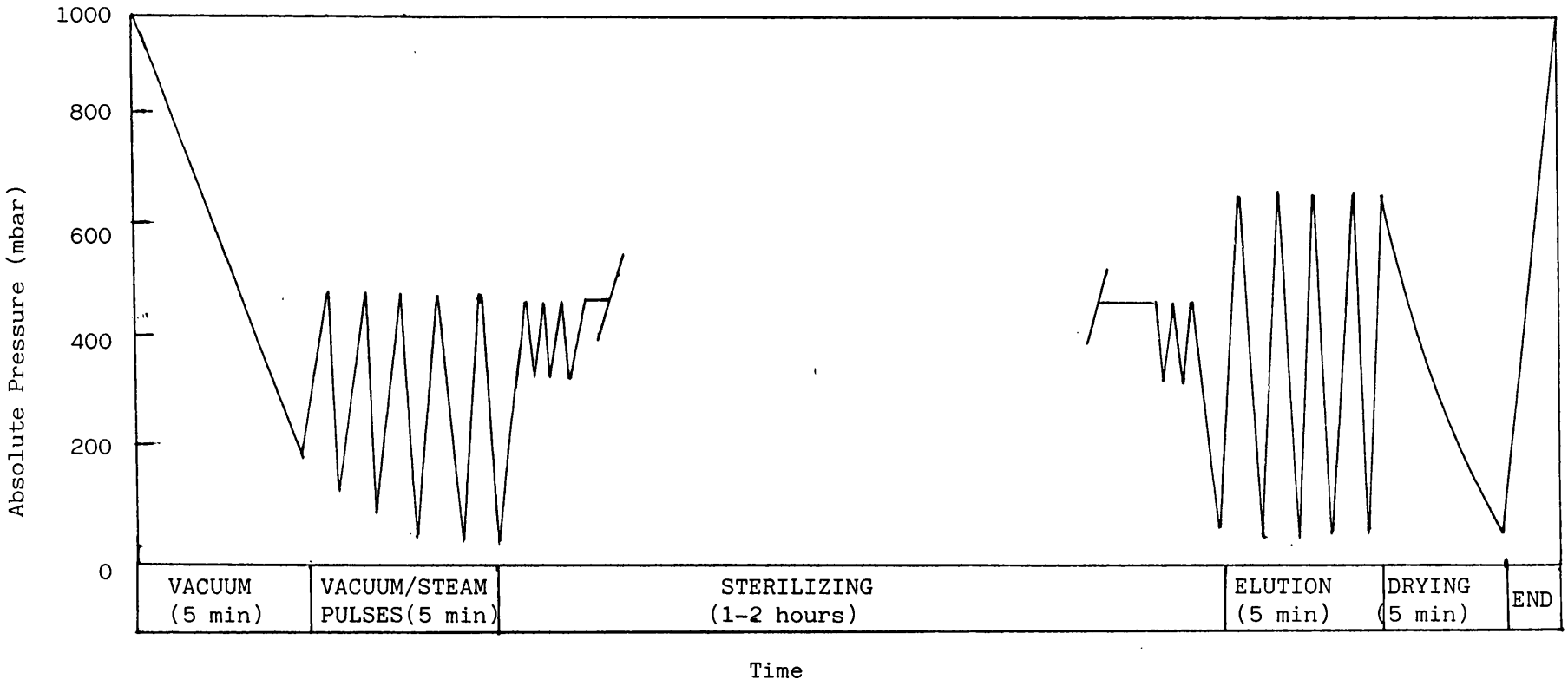


Fig. 8 Typical Commercial LTSF Operating Cycle. (From Drayton-Castle LTSF Sales Brochure)

1.13.2 Process Variables and their Control

The efficacy of Low Temperature steam with Formaldehyde sterilization is influenced by the formaldehyde concentration, temperature, water content in the bacterial spores and the duration of exposure.

1.13.2.1 Relative Humidity

A high relative humidity (RH) is necessary for rapid inactivation of microorganisms. Values between 70-90% (133) and 75-100% (138) have been reported as desirable for reliable formaldehyde sterilization. LTSF sterilizers should be such that RH is accurately controlled. Too much water vapour in the chamber can cause formaldehyde polymerisation thereby reducing the sporicidal activity on the microorganisms.

1.13.2.2 Vacuum

A high vacuum in the chamber is necessary to allow penetration of steam and formaldehyde into narrow bore tubings and layers of fabric. Subatmospheric conditions enable the maintenance of steam temperature by pressure control. LTSF cycles often operate at pressures between 100 and 400 mbar absolute. The UK LTSF protocol operates at 350 mbar ± 30 mbar. Early cycle failures were often associated with poor penetration of narrow-bore tubing (125, 126).

1.13.2.3 Temperature

The ability of formaldehyde to polymerise at temperatures below 80°C often gives rise to problems in LTSF cycles. One of the commonly cited disadvantages in the use of low temperature steam with formaldehyde is the need to maintain temperature of the nature of 80°C to prevent polymerisation. This temperature may still be damaging to some highly thermolabile objects and materials. The use of uniformly heated chamber jackets and jacketed doors reduces the risk of condensation and subsequent polymerisation at operating temperatures below 80°C. Jacket temperatures are often set 1-5°C above operational temperatures to minimise formation of superheat conditions. Operational tempreatures between 65° and 80°C have been used for LTSF sterilization (139). The accepted temperature in the UK is 73° ± 2°C although there was no rationale in the choice of the temperature other than that successful and reproducible cycles were obtained in a modified apparatus operating at that temperature (140, 141). The necessity to sterilize more delicate thermo-sensitive equipment, like electronic equipment, requires the use of lower operational temperatures of the nature of 55°C (133).

1.13.2.4 Formaldehyde Concentration

There seems to be little published data on the effect of formaldehyde concentration on the efficacy of the LTSF process. Hoxey (22) demonstrated that spores of B. stearothermophilus NCIB 8224 became more sensitive to LTSF at 80°C as the formaldehyde

concentration was increased from 6 mg l^{-1} to 20 mg l^{-1}. However, the observed increase in sensitivity was not consistent with equal incremental formaldehyde concentration increases for concentrations between 20 mg l^{-1} to 27 mg l^{-1} (22).

Concentrations between 3.3 mg l^{-1} and 100 mg l^{-1} have been used in commercial LTSF protocols (2). The UK LTSF protocol recommends the use of 25 mg l^{-1} of formaldehyde. Handlos (142) reported that the level of formaldehyde residuals in LTSF processes was dependent on the formaldehyde concentration. Therefore the minimum effective formaldehyde concentration is always desirable to reduce residual levels.

1.13.2.5 Exposure Time

Exposure times used in LTSF sterilizations are shorter than those required for ethylene oxide (EO) sterilization. The official time of exposure for the UK LTSF protocol is 2 hours although shorter periods or longer periods may be necessary depending on the nature of the material being sterilized and the efficiency of the LTSF apparatus (138, 139). The complex interrelationships of the many variables involved in LTSF processes necessitate the use of biological indicators to integrate all these variables in evaluating the antimicrobial efficiency of the process. Physical and chemical means alone are inadequate.

1.14 MECHANISM OF LTSF INDUCED LETHALITY IN BACTERIAL SPORES

The exact mechanism by which LTSF inactivates microorganisms including viruses is not known. However it is generally accepted that LTSF utilises the synergistic sporicidal effect of the latent heat of LTS and the formaldehyde monomer (135). LTS is thought to bring about its antimicrobial effects by the denaturation of important molecules like proteins (143) and spore enzymes (24) due to the breakdown of hydrogen bonds (143). Single strand (145) and double strand (146) chromosomal breaks have been shown to occur in vegetative organisms and in B. subtilis spores (147) exposed to moist heat. Cortical and plasma membranes and spore coats have been shown to be disrupted by moist heat (148, 149). It is very likely that LTS induces the same effects on bacterial spores as moist heat in disrupting the integrity of the intact bacterial spore.

As an alkylating agent, formaldehyde will react with primary amide and amino groups in protein molecules (150) by binding protein to give a denser, less permeable structure (151). Purine nucleotides e.g. guanine and adenine will form stable reaction products with formaldehyde (152) but pyrmidine nucleotides do not form stable reaction products. It also acts as an alkylating and mutagenic agent reacting with carbonyl, thiol and hydroxyl groups (153). Formaldehyde reacts to a greater extent with nucleic acids than proteins (154) and with RNA more than with DNA (154, 155). Auerbach et al. (153) reported that formaldehyde will not

react with tightly wound nucleic acids but will react readily with unwound molecules.

It is proposed here that the latent heat of LTS disrupts the spore integrity facilitating penetration of formaldehyde to the reactive sites exposed by the heat. Monomeric formaldehyde is the reactive species and temperature increases are synergistic with formaldehyde, since the monomer-polymer equilibrium is shifted to the monomeric form (156).

The bactericidal power of formaldehyde is superior to ethylene oxide but it has a weak penetrating power and has been considered to be a surface bactericide (141). Alder argues that to be effective, the gas has to dissolve in a film of moisture surrounding the spore and for this reason high relative humidities of the order of 70-90% are required (133).

Trijillo and David (123) attributed the sporicidal activity of formaldehyde to an irreversible reaction involving the nucleic acids and also reported that formaldehyde caused sporostasis due to a reversible inhibition of germination. However, these early claims of sporicidal activity were considered to be due to sporostasis (157) and recent studies by Spicher and Peters (158) support the claims of sporicidal activity of formaldehyde as due to a reversible inhibition of germination. These authors (158) reported that subsequent heating will reactivate spores apparently inactivated by formaldehyde. This reactivation phenomenon is an

area of current research to establish whether it also occurs after LTSF exposure (159).

1.15 FORMALDEHYDE TOXICITY

Acute toxic symptoms due to exposure to formaldehyde are irritation of the mucous membranes of the eyes, nose and throat. Continual exposure can also cause severe irriation of the conjunctiva and respiratory tract, and pulmonary oedema (160). As an alkylating agent, its reactions with proteins, nucleotides and nucleic acids have resulted in formaldehyde being classified as a mutagen. Formaldehyde has been reported to be mutagenic in Drosophila, yeast and bacteria (110) and to cause squamous cell carcinomas in rat nasal mucosa (161). There are no reported incidents of carcinogenicity or teratogenicity in human beings following exposure to formaldehyde. Formaldehyde is a normal metabolite involved in human metabolic pathways and cells survive in spite of the presence of chemically reactive and cytotoxic formaldehyde as a consequence of active enzymatic pathways for its removal (168). Therefore exposure to it is considered not to constitute a danger but is obviously undesirable (162). Allergic dermatitis has been reported (163) and although it is necessary to have the minimum of residual levels following sterilization, residual formaldehyde in fabrics and other materials might be of value in producing a self-disinfecting action on storage (162).

The majority of epidemiological evidence shows no increase

in mortality for all cancer or for cancers of any site in populations exposed to formaldehyde (164). Overall conclusions from the studies on formaldehyde in animals indicate that it presents a potential carcinogenic hazard in men. The size of the risks associated with exposure cannot be quantified but is very low (164). Most toxicological studies have been conducted on mice and rats which are susceptible to formaldehyde than man.

Precise advice on how to protect those exposed to formaldehyde from its long term effects must at the minimum comply with statutory requirements drafted in various countries where formaldehyde is used for various purposes. Most of these countries have defined the permitted maximum level of formaldehyde in the air at work places.

In Great Britain the permitted maximum level is 10 ppm (165) and in USA a ceiling of 5 ppm is set with an acceptable peak above the ceiling of 10 ppm for no more than 30 minutes in eight hours (166). In Denmark the permitted maximum is 1 ppm (167). As far as skin exposure is concerned, it should be relatively easy to avoid contact with formaldehyde by using suitable protective clothing.

CHAPTER 2

GENERAL APPARATUS, MATERIALS AND METHODS

CHAPTER 2. GENERAL APPARATUS, MATERIALS AND METHODS

2.1 GENERAL APPARATUS

2.1.1 Glassware

Grade B standard volumetric glassware was used throughout this work (Fisons Ltd.). Narrow neck, Erlenmeyer flasks (Pyrex) of 250 ml capacity were used for the cultivation of micro-organisms in liquid media. Test-tubes were rimless and of pyrex glass (150 mm x 16 mm) with metal caps (Oxoid). Clear medical flat bottles of 150 ml and 500 ml capacity (Fisons Ltd.) with metal caps and polypropylene caps respectively, were used for cold room storage of solutions and in the preparation of sporulation media.

Glass spreaders were prepared from 3 mm glass rods bent at an angle of about 60° to give a 70 mm handle and a 30 mm spreading portion.

2.1.2 Preparation of Glassware

Previously decontaminated glassware was washed using a dilute solution of Linkdet 704 (Link Chemicals Ltd.) and rinsed five times in tap water and three times in glass distilled water. After rinsing, the items were dried in an 80°C warm air oven (LEEC). The openings of glassware were covered with aluminium foil. Glass spreaders were packed in glass beakers and covered with

aluminium foil. All glassware was sterilised in a hot air oven operating at 160°C for 120 minutes (Griffin 200FL). Caps of 150 ml and 500 ml medical flats were packed into heat-sealed autoclave bags (DRG Ltd.) and autoclaved at 121°C for 15 minutes in a bench autoclave (Taylor Rustless Fittings Co. Ltd., Leeds).

2.1.3 Plasticware

Polystyrene petri-dishes were used generally for the performance of viable counts when the appropriate nutrient medium had been allowed to set in them. They were also used in the preparation of solid sporulation media. The Petri-dishes were supplied pre-sterilised (Sterilin Products Ltd.) and were of 10 cm diameter and single vented. Pre-sterilised 30 ml capacity polystyrene universals with polypropylene tops were used as sterile containers during processing of spore crops and for storage of working spore suspensions.

2.1.4 Semi-Automatic Pipettes

Semi-automatic, adjustable air displacement replicating pipettes were used throughout this work. They were of the following volume ranges.

Pipette	Serial No.	Volume Range	*Precision (Supplier's) Repeatability C.V. (%)
Pipetman P200	K 80 13421	50 - 200 µl	0.40 - 0.15
Pipetman P1000	M 84 12459	200 - 1000 µl	0.30 - 0.15
Pipetman P5000	L 84 17885	1000 - 5000 µl	0.30 - 0.16

*Manufacturer's precision repeatability, the closeness of agreement between the individual volumes, was calculated from

$$C.V.(\%) = \frac{SD \times 100}{\bar{v}}$$

where SD is standard deviation

and $\bar{v}$ is the mean value of all volumes measured with a minimum of 10 measurements.

The pipettes were checked gravimetrically by weighing replicate volumes of glass distilled water at 20°C. The balance chamber was humidified by exposing a petri-dish of distilled water overnight. The nominal volumes used included the ones that were commonly used throughout this work. For the calculation of the mean error, the weight of 1 ml of water at 20°C was taken to be 0.99823 g as quoted in Documenta Geigy (169). In all cases, the mean error, i.e. the closeness of agreement between the nominal weight and the mean weight of measured volumes, was between 0.007 and 1.58% (Appendix A1). The coefficient of variation in all cases was between 0.07% and 1.57%. The values are within the manufacturer's quoted values and represent the high degree of reproducibility and precision in their use.

2.1.5 Pipette Tips

Appropriate polypropylene tips for each pipette were decontaminated by autoclaving at 121°C for 15 minutes and washed in a dilute solution of Linkdet 704 (Link Chemicals Ltd.). The tips were then rinsed five times in tap water and three times in glass distilled water. They were dried in a warm air oven at 80°C (LEEC) and packed into heat sealed autoclave bags (DRG Ltd.). The tips were then sterilised by autoclaving at 135°C for 4 minutes in a porous loads sterilizer (Drayton Castle Ltd.).

2.1.6 "Swinnex" Filter Units

"Swinnex" filter units (Millipore Corporation, Massachussetts, U.S.A.) were cleaned as for polypropylene tips above. Appropriate pore size cellulose nitrate membrane filters were fitted and the units assembled with a few drops of water placed on the membranes. The assembled units were sealed in autoclave bags and sterilised by autoclaving at 121°C for 15 minutes. Before use, the units were tightened to avoid filtrate leakage.

2.1.7 Positive Pressure Membrane Filtration Apparatus

Clarification and filter sterilisation of large volume solutions that could not be autoclaved were performed using the Sartorius 100 mm stainless steel in-line filter holder (Sartorius

Instruments Ltd.) under laminar flow conditions. All openings of the cleaned apparatus were covered with aluminium foil and the whole apparatus sealed in a large heat sealed autoclave bag (DRG Ltd.). The apparatus was sterilised by autoclaving at 135°C for 4 minutes in a porous loads sterilizer (Drayton Castle Ltd.) 0.45 µm and 0.20 µm pore size cellulose nitrate filters of 100 mm diameter were used for clarification and sterilisation respectively. The membrane filters were sterilised in a bench-top autoclave at 121°C for 15 minutes in autoclave bags. The filters were then aseptically fitted into the stainless steel in-line filter holder inside the laminar flow cabinet prior to use. The first portion of the filtrate was always discarded and the filters visually checked at the end for any damage. A positive pressure of around 2 Bar was used.

2.1.8 pH Meter

A combined electrode pH meter (Philips PW 9418) was used to check the pH of all constituent aqueous solutions for the preparation of defined media. Standardised buffer solutions of pH 4.0, 7.0 and 9.2 (BDH Ltd.) were used in the routine calibration of the pH meter.

2.1.9 Weighing Balances

Oertling TP31 2-figure and Oertling Series 040 4-figure balances were used throughout this work.

2.2 MATERIALS

2.2.1 Chemicals

Analytical Reagent Grade chemicals were obtained from Fisons Ltd. and BDH Ltd. Sigma Chemical Co. provided chromatographically homogeneous amino acids. All vitamins were obtained from Sigma Chemical Co. and stored at -20°C.

2.2.2 Water

All water used for microbial purposes was glass distilled with pH ranging between 4.5 - 5.0. The glass distilled water was sterilised by autoclaving at 121°C for 15 minutes in 150 ml and 500 ml medical flat bottles. For the preparation of chemically defined media, the glass distilled water was freshly boiled and cooled to remove any dissolved carbon dioxide.

2.2.3 Nutrient Agar (NA)

Nutrient Agar (Lab-M Ltd.) was used throughout this work, unless specified, for the determination of spore viable counts and for the recovery of spores surviving an inactivation treatment. This was prepared by mixing 28 g of dry granules with 1 litre of glass distilled water. The medium was sterilised by autoclaving in 500 ml medical flats at 121°C for 15 minutes in a bench-top autoclave. After cooling to about 60°C, approximately 20 ml

aliquots of the molten agar were aseptically distributed into sterile petri-dishes and left standing to solidify. The plates were stored inverted at 4°C in the dark and used within seven days. Prior to use, the surfaces of agar were overdried at 37°C for an hour.

2.2.4 Tryptone Soy Agar (TSA)

Tryptone Soy Agar (Lab-M Ltd.) was prepared by mixing 37 g of dry granules with 1 litre of glass distilled water. The medium was sterilised as for Nutrient Agar above and the plates prepared and stored in like manner.

2.2.5 Tryptone Soy Broth (TSB)

Tryptone Soy Broth (Lab-M Ltd.) was used in the preparation of primary cultures of microorganisms. 30 g of dried granules were mixed and dissolved in a litre of glass distilled water. 100 ml portions of the solution were distributed into 150 ml medical flats and sterilised by autoclaving at 121°C for 15 minutes. Sterile TSB was stored at room temperature for up to three months.

2.3 MICROBIAL METHODS

2.3.1 Determination of Total Count of Spore Suspensions

A platelet counting chamber with improved Neubar ruling on a

silver background (Hawksey Ltd.) was used to determine the Total Count of spore suspensions. The method recommended by Cook et al. (170) was adopted. A drop of a suitable spore suspension, to give counts between 6 and 12 of the phase-bright spores per small square, was introduced under the coverslip using a sterile Pasteur pipette. The preparation was allowed to settle for 10 minutes while the drop spread over the grid by capillary action. The slide was then examined under phase-contrast microscopy at x400 magnification (Wild, Switzerland). The number of phase-bright spores in at least 80 small squares counted diagonally across the grid was recorded. The Total Count was calculated as follows:

$$\text{Total count} = \bar{x}.d.4 \times 10^6 \text{ spores/ml}$$

where $\bar{x}$ = mean count per small square

d = dilution factor of the spore suspension

An example of a quintuplicate total count determination is shown in Table 1 which summarises the results obtained for a B. stearothermophillus NCIB 8224 spore suspension (B6F). The coefficient of variation of 2.9% is within acceptable limits and indicates that the method is suitable for use in these studies.

2.3.2 Determination of Viable Count of Spore Suspensions

The spread plate technique was used to determine the viable count of spore suspensions. Ten-fold serial dilutions of stock suspensions were carried out using sterile distilled water (0.5 + 4.5 ml or 1 ml + 9 ml) to terminate with suspensions containing

Table 1. Data for the quintuplicate determination of the Total Count of B. stearothermophilus NCIB 8224 spore suspension. Batch 6F

Sample No.	Dilution Factor	Mean Count per Small Square	Total Count/ml	Mean Total Count/ml
1		9.81	3.92×10^8	
2		9.60	3.84×10^8	
3	10^{-1}	9.25	3.70×10^8	3.77×10^8
4		9.13	3.65×10	
5		9.38	3.75×10^8	

Mean Total Count (x) = 3.77×10^8/ml

Standard deviation (S.D.) = 1.08×10^7

Coefficient of Variation (C.V.) = 2.9%

approximately 250 - 1000 spores/ml. Suspensions were thoroughly mixed at each dilution using a vortex mixer (Whirlimix, Fisons). 0.2 ml aliquots of the final dilution were aseptically transferred and spread on the surface of each of five overdried plates of recovery medium. Sterile glass spreaders were used to spread the suspension taking care not to touch the petri-dish walls. The plates were left standing for 5 minutes and then incubated, inverted at the appropriate incubation temperatures for 48 hours. Colony forming units were then enumerated. Assuming each colony forming unit arose from a single spore, and with knowledge of the dilution factor, the viable count of each series was calculated. Data displayed in Table 2 are for quintuplicate determinations of the viable count of a B. stearothermophilus NCIB 8224 spore suspension batch (B6F). The samples 1-5 were taken on each of five consecutive days. Incubation was at 56°C for 48 hours. The Analysis of Variance performed on these data is also given in Table 2.

The Analysis of Variance displayed in Table 2 shows that the variation between samples is significantly greater than the variation within replicates of a single sample. However, since the overall coefficient of variation (1.7%) is within statistically acceptable limits, the spread plate technique can be considered suitable for determining the viability of treated and untreated bacterial spores.

Table 2. Data for the quintuplicate determination of Viable Count for a B. stearothermophilus NCIB 8224 spore suspension Batch B6F with Analysis of Variance of the data.

Sample	Dilution factor	Colony Counts	Mean Colony Count	Mean Viable Count/ml
1		132,130,134,134,131		
2		129,129,131,132,129		
3	1×10^5	131,131,130,128,129	129.8	6.50×10^7
4		128,134,132,127,129		
5		130,126,125,127,127		

Source of variance	Degrees of Freedom	Sum of Squares	Mean Square	F
Between samples	4	68.4	17.1	3.7
Within samples	4	2.0	0.5	0.1
Residual	16	73.6	4.6	
Total	24	144.0		

Overall coefficient of variation (C.V.) = 1.7%

$F(4, 16)\ P_{0.05}$ = 3.01

2.3.3 Determination and Reproducibility of Germination Indices

Most bacterial spore populations have a fraction that shows a reluctance to germinate (171). Two stages are involved in the production of a visible colony: first the germination and emergence of a vegetative cell and second, the survival and outgrowth of the vegetative cell. These two phases may require different optimal conditions (170). The fraction that does not outgrow into colonies could be due to (a) non-viable spores, (b) viable spores that fail to germinate at conditions supplied or (c) the failure of the environment to support outgrowth and multiplication following the initiation of germination. The germination index, the ratio of the viable count to the total count expressed as a percentage, describes this phenomenon. The Germination Index (G.I.) value depends on the accuracy and precision of the methods used in evaluating total and viable counts of spore suspensions.

The value of the G.I. is species-specific and also governed by the recovery conditions. The reproducibility of total counts and viable counts has been demonstrated in Tables 1 and 2. Table 3 shows the reproducibility of five replicate G.I. determinations on a single batch of B. stearothermophilus NCIB 8224 (B6F) and on three batches of the same strain, using two recovery media (NA and TSA) after incubation at 56°C for 48 hours.

The coefficient of variation observed within a spore batch and between spore batches is within acceptable limits and thus the

Table 3. Reproducibility of G.I. for B. stearothermophilus NCIB 8224 (Incubation at 56°C for 48 hours)

a) Reproducibility within a single batch (B6F)

Replicate	Recovery Medium	
	NA	TSA
1	18.03	15.45
2	16.82	15.40
3	17.29	16.05
4	16.87	15.61
5	17.03	15.10
Mean	17.21	15.52
S.D.	0.49	0.35
C.V.	2.85%	2.25%

b) Reproducibility between batches

Batch	Recovery Medium	
	NA	TSA
B6F	17.21	15.52
B11F	17.00	15.91
B26F	16.90	15.83
Mean	17.04	15.75
S.D.	0.16	0.21
C.V.	0.93%	1.33%

Germination Index is one of the parameters that will be used to characterise bacterial spore strains. The dependence of the G.I. value on recovery conditions is exemplified by the different G.I. values obtained for the same strain when recovered from the two different recovery media (NA and TSA). For spores of Bacillus stearothermophilus NCIB 8224, the G.I. obtained using TSA is around 10% lower than that obtained using NA under the same incubation conditions. Similar results were observed with spores of other test strains and thus Nutrient Agar was used as the recovery medium in all subsequent experiments. 48 hours was selected as the incubation time since extended incubation up to 5 days produced no significant increase in colony count.

CHAPTER THREE

PRODUCTION OF BACTERIAL SPORES FROM CHEMICALLY DEFINED SPORULATION MEDIA

3.1 INTRODUCTION

Spores of Bacillus species have been widely used in testing and validating various sterilization processes (172). It is desirable that the media used to produce these spores should provide spore crops showing minimal batch to batch variation in their rate and extent of growth and in their resistance to physical or chemical agents. Chemically defined media (CDM) have been successfully used to produce spore crops of high reproducibility in characterisation parameters (1, 173, 174). The advantage of CDM has also been recognised by their use for the production of vegetative cells of reproducible characteristics in tests evaluating hospital disinfectants (175) and preservative efficacy in eye drops and contact lens solutions (176).

Chemically defined media can further be defined in terms of the nature of the specific nutrient whose depletion limits the exponential vegetative growth and induces sporulation (24, 34, 111) and also modifies the spore characteristics (144, 177).

The experiments described in this chapter compare the characteristics of selected spores when grown on two different chemically defined sporulation media. The CDM selected were:

1. SSMAVIT medium, first described by Hobbs (178), is based on the medium developed by Lazarini and Santangelo (179). This medium was successfully used by Hoxey (22) to produce

potential LTSF biological indicator organisms. The media excelled those of DeGuzman (180) and Anderson and Friesen (181). SSMAVIT, though chemically defined, is complex with glucose limiting at 8.33 mM. The medium can be used as solid or liquid.

2. Carbon-Limited (C-Ltd) medium was first developed by Steele (182) from a medium reported earlier by Lee and Brown, (144). It is a simpler CDM than SSMAVIT and is currently being investigated at the Department of Health LTSF Reference Laboratory, Luton, as the medium for production of spores to be used as biological indicators for LTSF processes (182).

In the production of spores for biological indicator development, the CDM of choice is the one that results in a high yield of spores with little debris (vegetative and sporangia) and requiring the minimum cultivation time. The resultant spores should also be of a high degree of reproducibility in high G.I. value and in high resistance to physical or chemical insult, relative to the most likely resistant contaminating organism in the load for a specific sterilization process.

It is important for spore suspensions to be free of sporangia, vegetative cells, media debris and nutrients carried over from the sporulation media because these can influence spore characteristics. A number of cleaning methods have been described.

The simplest and commonly used method is that of Long and Williams (183) based on repeated centrifugation and washing. Density gradient centrifugation (DGC) has been used for the separation of germinated from non-germinated spores but is a prolonged and complex method (184). Sachs (185) reported on a method based on a differential partitioning of spores and vegetative cells to polyethylene glycol and phosphate buffer respectively in a two phase separation system. Lysozyme has also been employed in lysing vegetative cells and debris in cleaning of spore suspensions (186). Ultrasonic and sonic waves have been used in the past to eliminate vegetative cells from spore suspensions and to break up spore aggregates (187) but this method is not advisable for use in spore suspensions to be used subsequently in heat resistance experiments since the treatments have been reported to reduce heat resistance markedly (188). The two-phase separation method of Sachs (185), the lysozyme treatment of Finley and Fields (186) and the density gradient method of Prentice *et al*. (184) all involve the use of materials that might affect spore characteristics. For this reason, the simple method of Long and Williams (183) was adopted in the studies reported in this thesis.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial Strains

i. *Bacillus stearothermophilus* NCIB 8224 and NCIB 10814. These strains were originally obtained from the National

Collection of Industrial Bacteria, Torry Research Station, Aberdeen. These strains were selected for further study as potential biological indicators for LTSF sterilization from an initial screening of 20 Bacillus species (22).

ii. Bacillus stearothermophilus NCTC 10003, originally from the National Culture Type Collection, was supplied by the LTSF Reference Laboratory, Luton, U.K. This strain was originally used as a biological indicator for moist heat sterilization and is currently being considered as a potential LTSF indicator organism (182).

iii. Bacillus subtilis Trav. 5230, was obtained from Travenol Laboratories, Thetford, Norwich. This strain of Bacillus subtilis is well characterised and has been included for comparative purposes.

3.2.2 Preparation of SSMAVIT medium

The composition of the complete SSMAVIT medium is shown in Table 4. The medium was prepared by aseptically mixing the sterile Stock Solutions detailed in Table 5.

The ingredients for stock solution I were dissolved with gentle heat in about 1800 ml of freshly boiled and cooled distilled water. After dissolution, the pH of the solution was adjusted to 6.9 - 7.1 and the solution made up to volume. Clarification and

Table 4. Constituents of Complete SSMAVIT Medium

Constituent	Concentration Molar	% w/v
Potassium hydrogen phosphate	1.76×10^{-2}	0.24
Dipotassium hydrogen phosphate	3.21×10^{-2}	0.56
Tri-sodium citrate	3.20×10^{-3}	0.10
'Tris' buffer base	2.98×10^{-2}	0.24
L-giutamic acid	3.02×10^{-2}	4.45×10^{-1}
L-tryptophan	1.22×10^{-4}	0.25×10^{-2}
L-methionine	1.68×10^{-4}	0.25×10^{-2}
L-histidine HCl	4.51×10^{-4}	0.70×10^{-2}
L-leucine	9.51×10^{-4}	0.70×10^{-2}
L-valine	7.68×10^{-4}	0.90×10^{-2}
Ferrous sulphate ($7H_2O$)	1.00×10^{-5}	0.03×10^{-2}
Magnesium chloride ($6H_2O$)	1.00×10^{-3}	2.03×10^{-2}
Zinc sulphate ($7H_2O$)	5.00×10^{-5}	1.00×10^{-3}
Calcium chloride ($2H_2O$)	1.00×10^{-3}	1.47×10^{-2}
Manganese chloride ($4H_2O$)	5.00×10^{-5}	1.00×10^{-3}
Thiamine HCl	4.45×10^{-7}	0.15×10^{-3}
Nicotinic acid	1.22×10^{-5}	0.15×10^{-3}
Biotin	4.09×10^{-8}	0.01×10^{-4}
Pyridoxal HCl	5.38×10^{-7}	0.09×10^{-4}
Folic acid	1.36×10^{-7}	0.06×10^{-4}
D-Glucose	8.33×10^{-3}	0.15

pH = 6.9 - 7.1.

sterilization were by positive pressure filtration using the 100 mm Sartorius Stainless Steel in-line apparatus (Chapter 2). 200 ml aliquots were stored in sterile 500 ml medical flat bottles at 4°C in the dark.

Solutions of the individual components for Stock Solutions II and III were prepared and sterilized by positive pressure filtration. In all cases, the first 50 ml of filtrate was discarded and subsequent 25 ml aliquots stored in 150 ml medical flat bottles at 4°C in the dark. The ferrous sulphate solution was freshly prepared as it readily oxidised to ferric sulphate solution when stored. Stock Solutions II and III were freshly prepared by aseptically mixing the required volumes of each solution as shown in Table 5.

Ingredients for the vitamin stock solution IV were dissolved in 450 ml of CO_2-free glass distilled water continuously stirred on a magnetic stirrer. The pH was adjusted to 6.9 and the solution made up to volume. Sterilization was by positive pressure filtration through a 0.2 µm pore size membrane filter. The first 5 ml of filtrate was discarded and subsequent 1.5 ml samples were sealed in sterile 2 ml glass ampoules and stored frozen at -20°C.

The complete SSMAVIT medium was prepared by aseptically mixing Solutions I-IV in the order given in Table 6 and adding 250 ml of sterile glass distilled water. Electrolyte Solution III was added in small portions to avoid precipitation. The preparation

Table 5. Composition of Stock Solutions for the Preparation of SSMAVIT Medium

Stock Solution I		Stock Solution II		Stock Solution III		Stock Solution IV	
Component	Quantity (g)	Component	Quantity (ml)	Component	Quantity (ml)	Component	Quantity (g)
K_2HPO_4	28	$FeSO_4.7H_2O$(mM)	50	$CaCl_2.2H_2O$(0.1 M)	50	Thiamine HCl	0.03
KH_2PO_4	12	$MgCl_2.6H_2O$(0.1 M)	50	$MnCl_2.4H_2O$(0.01M)	25	Nicotinic acid	0.3
tri-sodium citrate	5	$ZnSO_4.7H_2O$(0.01M)	25	Sterile Distilled water	to 250 ml	Biotin	0.002
'Tris'-buffer base	18	Sterile distilled water	to 250 ml			Pyridoxal HCl	0.018
L-glutamic acid	22.25					Folic acid	0.012
L-tryptophan	0.125					Distilled water to 500 ml	
L-methionine	0.125						
L-leucine	0.6						
L-histidine HCl	0.35						
L-valine	0.45						
D-Glucose	7.5						
Distilled water to	2000 ml						

was thoroughly mixed at each addition and 50 ml volumes of the liquid media were aseptically distributed into sterile 250 ml Erlenmeyer flasks.

SSMAVIT was also used as a solid medium. This was made by adding the mixed solutions (I-IV) to 250 ml of sterile molten agar. The agar component was prepared by mixing 7.5 g of agar granules (Lab-M Ltd.) with 250 ml of glass distilled water. The agar component was sterilized in 500 ml medical flats by autoclaving at 121°C for 15 minutes. After cooling, the solid agar was stored at 4°C and freshly melted when required. Approximately 30 ml of solid medium was aseptically distributed into sterile petri-dishes and allowed to set. Solid SSMAVIT medium could be stored at 4°C in the dark for up to seven days prior to inoculation.

Table 6. Preparation of Complete SSMAVIT Medium from Sterile Stock Solutions

Stock Solution	Quantity (ml)
Solution 1	200
Solution IV	1.25
Solution II	25
Solution III	25
Molten Agar of Sterile water	250

3.2.3 Preparation of Carbon-Limited Medium

The composition for the complete C-Ltd medium is shown in Table 7 and was prepared by aseptic mixing of the sterile stock solutions shown in Table 8.

Ingredients for the Carbon Source Solution 1, were dissolved and made up to volume using freshly boiled and cooled distilled water. Sterilization was by positive pressure filtration through a sterile Swinnex unit fitted with a 0.2 m pore size membrane filter. 100 ml aliquots were stored at 4°C in the dark.

The buffer system, Solution II was prepared by dissolving the ingredients and making up to 500 ml using freshly boiled and cooled distilled water. 100 ml aliquots were distributed into 150 ml medical flats and sterilized by autoclaving at 121°C for 15 minutes.

The electrolyte solution, Solution III, was freshly prepared immediately before use by dissolving the ingredients and making up to volume using freshly boiled and cooled glass distilled water. The solution was filter sterilized using a sterile Swinnex unit fitted with a 0.2 µm pore size filter membrane. The solution could not be stored because the ferrous sulphate readily oxidised to ferric sulphate on standing.

Table 7. Constituents of Complete Carbon-Limited Medium

Constituent	Concentration		
	Molar	% w/v	g/litre
Glutamic acid	2.4×10^{-3}	3.53×10^{-2}	0.353
Glucose	1.0×10^{-3}	1.80×10^{-2}	0.180
Methionine	4.5×10^{-5}	7.00×10^{-4}	7.0×10^{-3}
Magnesium chloride ($6H_2O$)	1.8×10^{-5}	3.70×10^{-4}	3.7×10^{-3}
Manganese chloride ($4H_2O$)	1.5×10^{-5}	3.0×10^{-4}	3.0×10^{-3}
Calcium chloride ($2H_2O$)	5.5×10^{-5}	8.1×10^{-4}	8.1×10^{-3}
Ferrous sulphate ($7H_2O$)	1.0×10^{-5}	2.8×10^{-4}	2.8×10^{-3}
Nicotinic acid (sodium salt)	6.0×10^{-7}	9.0×10^{-6}	9.0×10^{-5}
Thiamine HCl	2.5×10^{-8}	8.0×10^{-7}	8.0×10^{-6}
Biotin	8.0×10^{-10}	2.0×10^{-8}	2.0×10^{-7}
disodium hydrogen phosphate	1.76×10^{-2}	2.498×10^{-1}	2.498
Potassium dihydrogen phosphate	7.3×10^{-3}	9.93×10^{-2}	0.993

pH = 6.9 - 7.1

Table 8. Composition of Stock Solutions for the Preparation of Complete Carbon-Limited Medium

Solution 1: Carbon Source		Solution II: Buffer System		Solution III: Electrolytes		Solution IV: Vitamins	
Component	Quantity (g)	Component	Quantity (g)	Component	Quantity (g)	Component	Quantity (ml)
L-Glutamic Acid	1.765	K_2HPO_4	4.965	$MgCl_2.6H_2O$	3.7	Thiamine (8 x 10-4% w/v)	10 ml
D-Glucose	0.900	Na_2HPO_4	12.49	$MnCl_2.4H_2O$	3.0	Nicotinic (9 x 10-3% w/v)	10 ml
Methionine	0.035	Distilled water	to 500 ml	$CaCl_2.2H_2O$	8.1	Biotin (2 x 10-5% w/v)	10 ml
Distilled water	to 500 ml			$FeSO_4.7H_2O$	2.8	Distilled water	to 40 ml
Distilled water	to 500 ml						

The vitamin Solution IV was prepared by mixing the individual ingredient volumes and making up to 40 ml using freshly boiled and cooled distilled water. Sterilization was by Swinnex filtration (0.2 μm). 4.5 ml aliquots were sealed in sterile 5 ml glass ampoules and stored at -20°C.

The complete C-Ltd medium was prepared by aseptically mixing the stock solutions in the order shown in Table 9 and adding the mixture to an equal volume of molten sterile 3% agar (Oxoid No. 3). The medium was then distributed into sterile petri dishes and allowed to set. Solid medium was stored at 4°C in the dark and used within 7 days.

Table 9. Preparation of Complete Carbon-Limited Medium

Stock Solution	Quantity (ml)
Solution I	100.0
Solution II	100.0
Solution III	0.5
Solution IV	4.0
Sterile distilled water	to 500
Sterile 3% Oxoid No. 3 Agar	500

3.3 CULTIVATION OF SPORE CROPS

3.3.1 Preparation of Inocula

12-18 hr primary vegetative cultures grown in Tryptone-Soy-Broth (TSB), unshaken at appropriate temperatures were distributed into sterile 30 ml plastic Universals (Sterilin) and centrifuged at 4000 rpm x 15 minutes (MSE Chilspin). The vegetative pellet was suspended in the liquid form of sporulation medium and the process repeated twice. The final clean vegetative suspension, free of complex TSB medium, was then used to inoculate the sporulation media. 1 ml (ca $2 \times 10^{8-9}$ cells) was used to inoculate 50 ml liquid SSMAVIT medium in 250 ml Erlenmeyer flasks plugged with sterile sponges. The flasks were incubated at 56°C (37°C for B. subtilis Trav 5230) without shaking to limit aeration. For solid media, 1 ml was spread on the surface of overdried media (37°C x 60 mins) and left standing for 15 minutes before the excess was aspirated off. Solid media plates were incubated at appropriate temperatures in humidified environments. Daily phase-contrast microscopic examinations of the sporulation cultures were performed to assess growth and sporulation.

3.3.2 Harvesting

Spore crops were harvested off solid media by aseptically flooding the surfaces with 5 ml ice cold sterile glass distilled water. The spores were detached using sterile glass spreaders, care

being taken not to disrupt the agar. The spore suspensions from all solid media inoculated from a single primary culture constituted a batch and were distributed into sterile 30 ml plastic Universals (Sterilin).

Liquid SSMAVIT media containing spores were distributed between several sterile 30 ml plastic Universals (Sterilin) and centrifuged at 4000 rpm for 10 minutes at 4°C (MSE Chilspin). The supernatants were discarded and all the spore containing pellets, arising from a single primary inoculation and constituting a batch, were aseptically transferred and resuspended in sterile glass distilled water in a single sterile 30 ml plastic universal (Sterilin).

The crude spore suspensions containing some vegetative cells and sporangia were stored at 4°C for cleaning.

3.3.3 Cleaning of Crude Spore Suspensions

The crude spore suspensions were centrifuged at 4000 rpm for 20 mins (MSE Chilspin) and the supernatant which was mostly vegetative cells and debris, was aspirated off without disturbing the spore pellet. The pellet was suspended in 20 ml of sterile glass distilled water and the process repeated at least five times until the suspension consisted of at least 95% phase bright spores. In some preparations showing large intractible spore aggregates, a final spin of 2000 rpm for 2 minutes was performed and the

supernatant retained. The clean suspensions contained mostly single spores and were adjusted to contain 10^8 - 10^9 spores/ml. The final suspensions were stored in sterile 30 ml plastic Universals at 4°C in the dark. 1.5 ml aliquots from each spore batch were sealed in 2 ml sterile ampoules and stored at -20°C as reference organisms.

3.4 PRODUCTION AND CHARACTERISATION OF SPORE CROPS

Each of the test strains was cultivated on solid SSMAVIT, liquid SSMAVIT and Carbon-Limited media. Daily microscopic examinations were performed to assess growth, percentage sporulation and the time taken to attain maximum sporulation. The ease of harvesting and cleaning was also determined. Finally, the Germination Index (G.I.) for spores from each of the strains was calculated. The results are summarised in Table 10.

3.5 DISCUSSION

It is well documented that conditions during sporulation can greatly influence spore characteristics (34. 177). The two chemically defined sporulation media considered in the investigation both supported bacterial growth and enabled sporulation of the selected bacterial strains. However, the extent of growth and sporulation differed from strain to strain and also depended on the medium. The results displayed in Table 10 show a diversity of the characteristics under investigation. The comparison of the characteristics was based on: (a) the influence

Table 10. Table to Show Extent of Growth yield, Incubation Time, Ease of Harvesting and Cleaning and Germination Indices of Spores of the Selected Strains of Bacillus Species Produced on Chemically Defined Media

	Solid SSMAVIT				Liquid SSMAVIT				C-Ltd			
Characteristic	NCIB 10814	NCIB 8224	NCTC 10003	Trav 5230	NCIB 10814	NCIB 8224	NCTC 10003	Trav 5230	NCIB 10814	NCIB 8224	NCTC 10003	Trav 5230
Extent of Growth*	+++	++	++	+++	+	++	+	++	++	+++	+++	++
Yield (% sporulation)	80	70	50	90	10	10	35	85	45	95	90	50
Maximum incubation (days)	7	7	3	4	8	9	3	7	4	2	2	4
Ease of Harvesting and Cleaning**	X	XX	XX	XX	XXX	XXX	XXX	XX	XX	X	X	XX
G.I. (%)	17	25	83	5.7	8	17.2	61	4.5	3	68	78	10.5

* (+) Low Growth, (++) Intermediated Growth (+++) Maximum Growth

** (X) 1-5 washes (XX) 5-10 washes (XXX) over 10 washes

of one composition of medium in either solid or liquid form using SSMAVIT medium, and on (b), the effect of different media compositions, SSMAVIT and C-Ltd both in solid form.

Amino acids are important and necessary for the provision of free energy necessary for vegetative cell growth (189). Several vitamin requirements including biotin, folate, p-aminobenzoate, thiamine and nicotinate have also been demonstrated as growth stimulatory in sporulating bacteria (189). C-Ltd medium has two amino acids present, L-glutamic acid and L-methionine, both at considerably less concentrations than those in SSMAVIT medium (see Chapter 4, Section 4.5). SSMAVIT medium, although chemically defined is very complex with abundant amino acids. C-Ltd medium also contains fewer vitamins with each at a lower concentration than in SSMAVIT medium. Growth was observed to be better on the simple Carbon-Limited medium and this indicates that simple chemically defined sporulation media are capable of producing good growth resulting in high spore yields.

Inoculum size, pH control and optimal glucose concentrations to 17 g/dm^3 have been reported to increase spore yields (61). Based on a common primary inoculum size of 1 ml vegetative cell suspension containing around $2 \times 10^{8-9}$ cells/ml, solid sporulation media seemed to produce a larger spore yield than liquid media. Ordal (190) and Halvorson (191) reported an increasing demand for oxygen as aerobic cells sporulate to sustain the endogenous changes leading to sporulation. Solid media provide a larger surface area

for gaseous exchange than stationary liquid media. The depletion of oxygen in liquid media at the crucial moments could account for the consistent poor yields observed. Shaking the liquid medium to increase aeration was tried but tended to increase vegetative cell growth observed as thick stringy growths. Sporulation in such cases was often poor and prolonged. A high spore yield of 85% with sporulation in liquid SSMAVIT medium was observed for B. subtilis Trav 5230 which is in close agreement with a 90% yield reported with the same medium by Hoxey (22). B. subtilis Trav 5230 sporulated exceptionally low on C-Ltd medium 50% but sporulated with high yields on solid SSMAVIT 90%, Anderson's medium 80% and De Guzman's medium 90% (22).

pH has been reported as having little influence on vegetative growth as observed with studies on B. cereus T buffered at pH 6.4, 7.0 and 7.4 (192). Although the optimum pH for sporulation is generally equivalent to that for growth (34) sporulation however is more fastidious than vegetative cell growth with respect to pH (193). Therefore during sporulation, the pH value has a pronounced effect on the yield but not necessarily on the resistance characteristics of the spores (77). For the purpose of this study, all sporulation media were prepared at neutral pH.

Manganese and iron proportions have been shown to be critical in growth (194) and manganese has also been reported to be a stimulus of sporulation in B. stearothermophilus and other spore formers (195). Phosphates and sulphates are essential to support

growth and potassium to ensure maximum spore formation (196). Magnesium is present since it is necessary for cell wall and membrane synthesis (197). All these electrolytes are present in both the CDM under investigation at different concentrations but it seems the proportions used in C-Ltd medium are preferable for better spore yields. Concentrations in liquid and solid SSMAVIT are similar but formulation in the liquid phase seemed to decrease spore yields except with B. subtilis Trav 5230.

Commercially, a sporulation medium that produces a high spore yield in the shortest incubation period is preferable. The medium should also be as simple as possible to facilitate preparation and quality control. Based on these criteria, C-Ltd medium would be the medium of choice from those tested since it is less complex and performs better than solid SSMAVIT medium, and the extent of growth and sporulation on liquid SSMAVIT medium was invariably poor for the thermophilic strains.

Maximum incubation times varied from two days for B. stearothermophilus NCIB 8224 and NCTC 10003 on C-Ltd medium to nine days for B. stearothermophilus NCIB 8224 in liquid SSMAVIT. A possible explanation for the earlier sporulation onset observed with C-Ltd medium could be due to the fact that this medium has less nutrients in lower concentrations than SSMAVIT medium and assuming comparable logarithmic vegetative growth rates, early nutrient depletion would occur in C-Ltd medium and result in sporulation. This is in accordance with the observation of Murrell,

(198) that the most important external factors to trigger sporulation were the disappearance of nutrients from the medium and the accumulation of catabolites, temperature, pH and oxygen availability.

Crops harvested from solid media were relatively easier to clean with strains of high sporulation requiring the minimum of washing to free them of vegetative cells, sporangia debris and immature spores. It is expected, and the results obtained with liquid SSMAVIT support this, that growth resulting in low spore yield should result in problems with spore cleaning because of large amounts of vegetative cells and sporangial debris present.

There is no definite pattern in G.I. values obtained for the strains on different sporulation media. The reproducibility of G.I. determination has been demonstrated (Table 3). As expected the results obtained show that for a given strain different sporulation media produce spores with different G.I. values. Baylis et al. (199) have shown how different sporulation media can produce spore crops of equal total counts but different viabilities and this is supported by the differing G.I. values obtained here.

Recovery media and conditions will affect germination and outgrowth of spores but in this study the same nutrient recovery medium (Nutrient Agar), was used to determine viability at 56°C for B. stearothermophilus strains and 37°C for B. subtilis Trav 5230. The difference in G.I. therefore might be explained in terms of

different extents of germination and outgrowth which are influenced by the sporulation media composition. Germination Index has long been considered as an intrinsic property of bacterial spores which can only be modified to an extent by sporulation conditions (178). The small differences in the magnitude of G.I. values for the same strain under different sporulation environments seem to be in accordance with the consideration. Gorman et al. (52) reported that in situations when maximum viability is desirable, this could be achieved by the application of ultrasonic energy and sublethal heat and chemical treatments e.g. peracetic acid and hydrogen peroxide when interference with the spore coat permeability might lead to promotion of germination.

In conclusion, the simple Carbon-Limited medium produced spores of B. stearothermophilus NCIB 8224 and NCTC 10003 in high yields, within two to four days incubation. These spores also exhibited high G.I. values desirable for organisms to be used as biological indicators. Should these strains possess the other necessary properties of an ideal monitor, then C-Ltd is recommended for the production of thermophilic spores for process monitoring.

CHAPTER 4

DETERMINATION OF RESISTANCE OF SPORES OF SELECTED *BACILLUS* STRAINS TO INACTIVATION BY MOIST HEAT AT 110°C

CHAPTER 4

4.1 INTRODUCTION

Heat resistance is probably the most studied and reported characteristic of bacterial spores. Bacterial spores are often considered the most heat resistant of all microorganisms, more so than any known pathogens. Because of this they would make the best marker organisms for validation of sterilization cycles and to monitor uniformity of processing conditions.

The previous chapter reported on the characterisation of the selected strains grown on both liquid and solid CDM in terms of their growth and sporulation, harvesting and cleaning and germination indices. Liquid SSMAVIT medium produced smaller spore yields that were more difficult to process than those produced on the other two solid CDM. Spores produced from solid SSMAVIT and C-Ltd media were therefore used for further investigations. High thermal resistance, at least higher than that of the most likely contaminating organisms, is a pre-requisite for any potential biological indicator organism. The work reported in this thesis was undertaken to study the response of potential biological indicator organisms to Low Temperature Steam and Formaldehyde (LTSF) treatment. Moist heat at temperatures around 70°C is one of the parameters involved in LTSF and a strain exhibiting maximum resistance to moist heat will be preferable as a monitor due to the higher safety margin offered. The choice of the marker organism

will however depend on the strain showing most of the desired characteristics for an ideal biological indicator.

Within the process of LTSF, there are a number of parameters that need to be integrated during process monitoring. It is therefore desirable for spores selected as biological indicators for LTSF sterilization to possess a high resistance to each parameter in isolation in order to confirm that a sporicidal combination has been produced. Spores exhibiting low resistance to moist heat, for example, would not be suitable as biological indicators for LTSF. Most LTSF sterilization protocols use temperatures around 70°C and therefore heat resistance studies to choose a biological indicator at similar temperature ranges, would be of direct relevance to the sterilization process. However, studying bacterial spore moist heat resistance at these temperatures presents considerable practical difficulties. In fact, spores of B. stearothermophilus strains often take exposure times in excess of 800 hr for the original spore population to be reduced by three log cycles when inactivated by moist heat at 73°C. The construction of survivor curves for such inactivation studies would present practical difficulties. Davies et al. (200) reported that moist heat inactivation at temperatures between 90° and 115°C significantly reduced spore viability within reasonable time spans. The experiments described in this chapter were undertaken to compare the moist heat resistance of the selected strains grown on different solid CDM as further characterisation. For the purpose of comparison of strains only, a temperature of 110°C was chosen as

survivor curves would be readily and reliably generated. This temperature is also within the practical range reported for sporal heat resistance studies (200).

Different techniques have been used to determine moist heat resistance of bacterial spores. Some of these methods can be summarised as below.

(i) Thermal death tube method, Bigelow and Esty (201);
(ii) Tank method of Williams et al. (202);
(iii) Thermal death time can, Townsend et al. (203);
(iv) Thermoresistometer method, Stumbo (204);
(v) Unsealed thermal death time tube method, Schmidt (205);
(vi) Capillary tube method, Stern and Proctor (206);
(vii) Pasteur pipette method, Scholefield and Abdelgadir (207);
(viii) Screw-cap tube method, Kooiman and Geers (208);
(ix) Solid heating block system, Malladis and Scholefield (209).

Most of these methods have problems of extended time lags during heating up and cooling and corrections to sampling times are therefore necessary. The thermoresistometer method (204) with instantaneous heating and cooling involves high capital cost. The screw-cap tube method (208) allows accurate heating without the need for correction of sampling times and avoids troublesome ampoule opening procedures but experimental results were not given with the description of the method. For the purpose of this

investigation, the thermal death tube method first reported by Bigelow and Esty (201) was used because of its simplicity, low capital cost and short heating-up and cooling down correction times. The disadvantages of this method are the fuming of oil at elevated temperatures and dirtiness. However, these were not considered hazardous since experiments were performed in a fume cupboard and appropriate protective gloves worn.

4.2 MATERIALS

4.2.1 Oil Bath

A thermostatically controlled oil bath (Grant Instruments) was used for the investigations described in this chapter. The heating was controlled by a 2 kW TEKAM TE4 thermostat (Techne, Cambridge Ltd.) which incorporated an electric stirrer to ensure even distribution of heat in the oil bath.

4.2.2 Thermocouples

Copper-constantan thermocouples (Comark, BS 4937J) were used for continuous temperature recording in determination of heating up and cooling down lag periods.

4.2.3 Recorder

A Servogor 120 continuous chart recorder (BBC Goerz

Metrawatt) was used to obtain the heating up and cooling down temperature-time profiles. The chart recorder was calibrated using boiling distilled water at 100°C and ice-distilled water at 0°C.

4.2.4 Heating Tubes

Sterile 2 ml freeze drying tubes (FBG Trident Ltd) were used to contain samples of bacterial spore suspensions for exposure to heat inactivation treatment. Prior to use, the tubes were immersed in boiling glass-distilled water for 30 minutes to leach out any removable ions, and sterilized by dry heat at 160°C for 2 hours.

4.3 METHODS

4.3.1 Determination of heating and cooling times

The heating (come-up) and cooling (quenching) times were determined using a copper-constantan thermocouple sealed into a 2 ml freeze drying tube containing 1.5 ml of sterile glass distilled water. The tube was immersed into the oil bath stabilized at 110°C and the temperature continuously recorded on the chart recorder until it stabilized at the set temperature. The tube was then removed and immediately immersed in crushed ice at 0°C and the cooling temperature again continuously recorded. The temperature-time profiles obtained are shown in Figure 9.

In the light of these results, a 90 second correction time

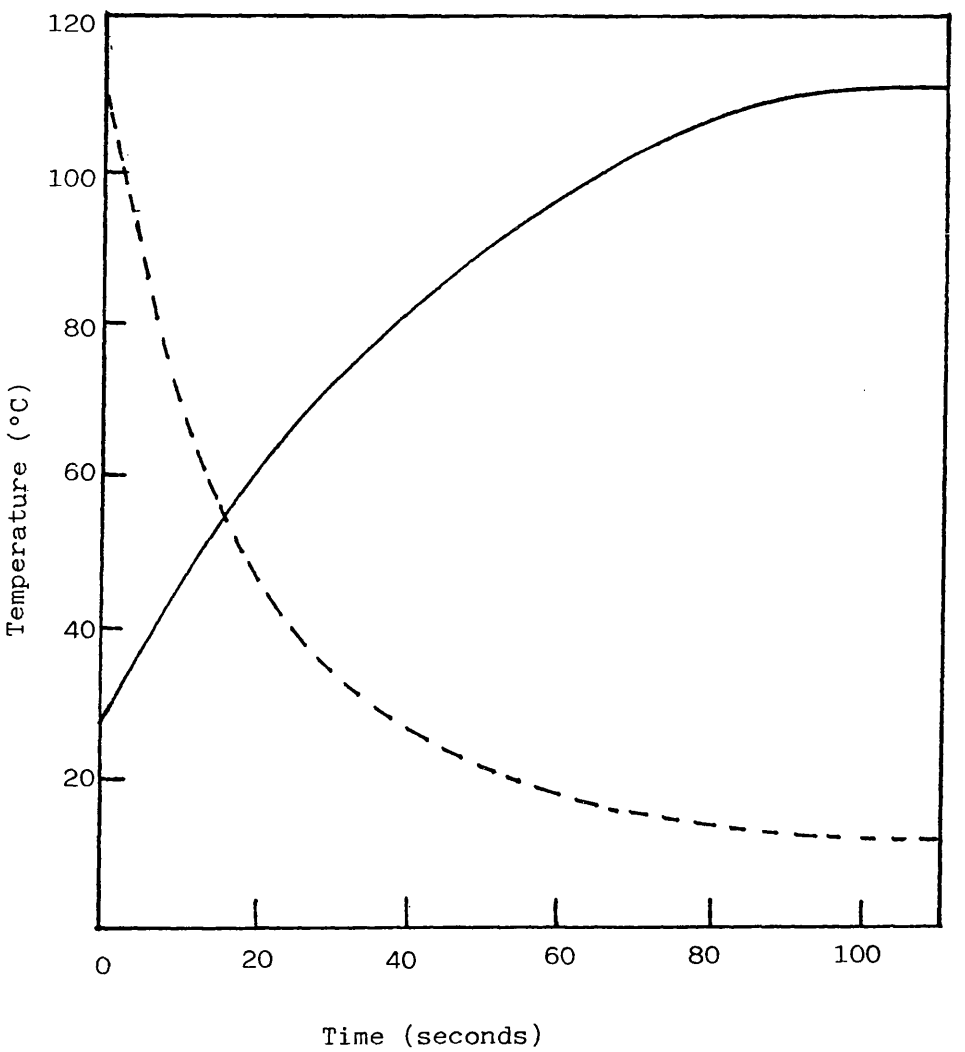


Figure 9. Heating up (—) and cooling (---) profiles for 1.5 ml water in freeze drying ampoules heated to 110°C in an oil bath and then cooled in crushed ice.

to sampling times was allowed for the heating up of the samples to the set temperature. The cooling time to below 90°C was found to be short (5 seconds). There was therefore no necessity to apply any correction.

4.3.2 Determination of spore heat resistance in aqueous suspension

Suitable dilutions were performed on the bacterial spore stock suspensions using filter sterilized, freshly boiled and cooled glass distilled water (pH = 6.2) to give suspensions containing around 5×10^6 spores/ml. 1.5 ml samples of these suspensions were aseptically sealed in sterile 2 ml freeze drying tubes.

The tubes were then immersed in the oil bath equilibrated at 110°C and removed at predetermined time intervals to be immediately immersed in crushed ice to terminate the heat lethality on the spores. After 2 minutes cooling, the tubes were aseptically file-opened and the viable counts of the contents of each tube determined as previously described (Section 2.3.2). Nutrient Agar was used as the recovery medium and plates were incubated at 56°C (37°C for B. subtilis Trav 5230) for 48 hours.

4.4 EXPERIMENTAL

4.4.1 Reproducibility of heat survivor curves

Bacterial spores intended for use in process validation and monitoring should show a high degree of batch to batch reproducibility (111). Different spore batches should therefore display standard resistance when exposed to the sterilizing conditions.

A comparison was made of the variation in heat resistance which occurred within and between two batches of spores of B. stearothermophilus 10814 (B2F and B9F) produced on solid SSMAVIT media. Triplicate determinations of the survivor curve for the spores from each batch were made following inactivation at 110°C, pH = 6.2 (Section 4.3.2).

Figures 10 and 11 show the results of the triplicate inactivations for the two batches B2F and B9F respectively when the surviving fraction, on a log scale, was related to the corresponding exposure time on the linear scale. The data expressed in Figures 10 and 11 reveal little variation between replicates of each batch. In both cases the inactivation is logarithmically linear but with some evidence of an initial lag period.

For survivor curves where there is evidence of a shoulder as displayed by the data in Figs. 10 and 11, if the time zero (100%)

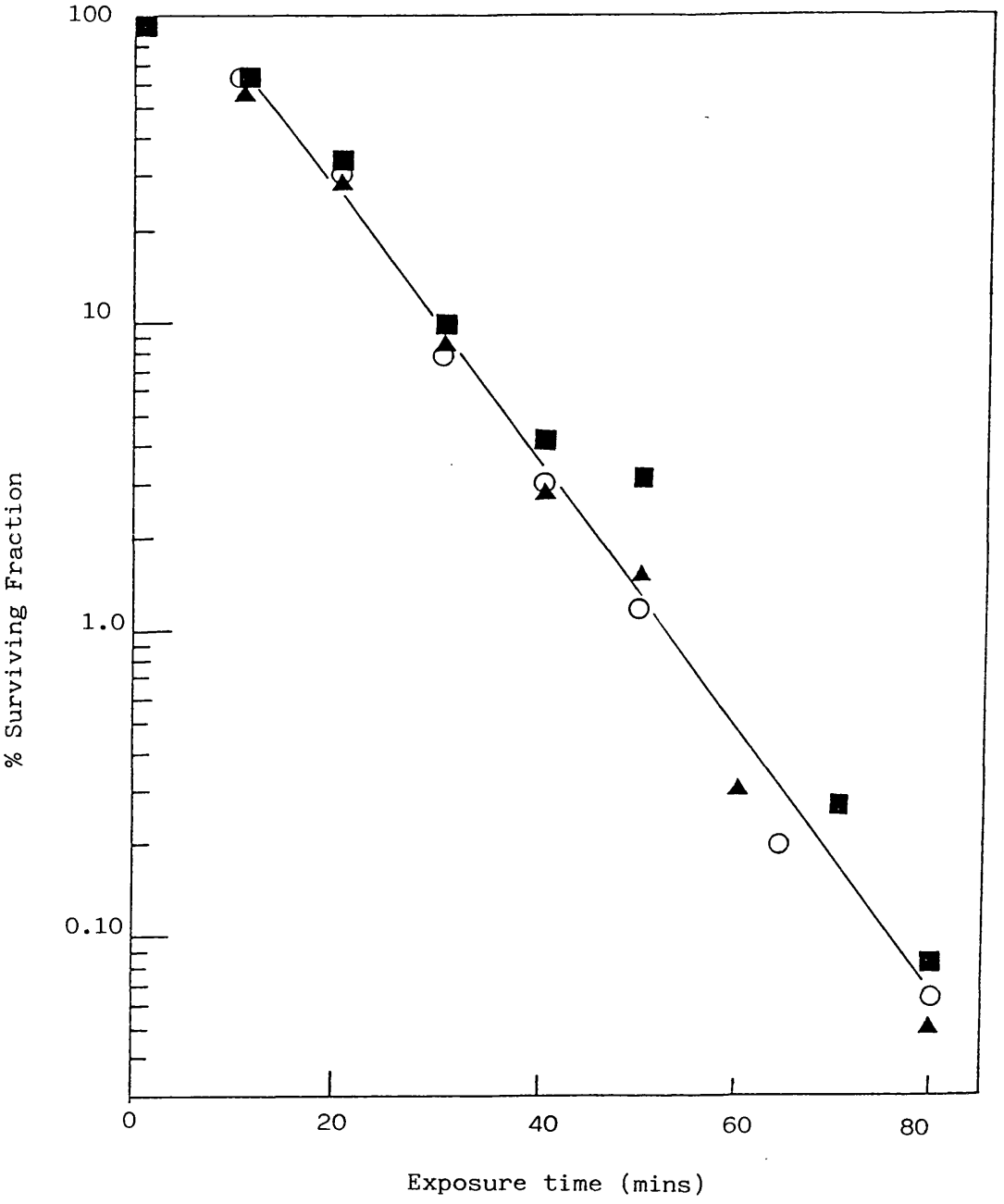


Figure 10. The inactivation by moist heat at 110°C of three replicates of spores of B. stearothermophilus NCIB 10814 (B2F) grown on solid SSMAVIT showing the survivor curve of best fit (R^2 = 0.9848)

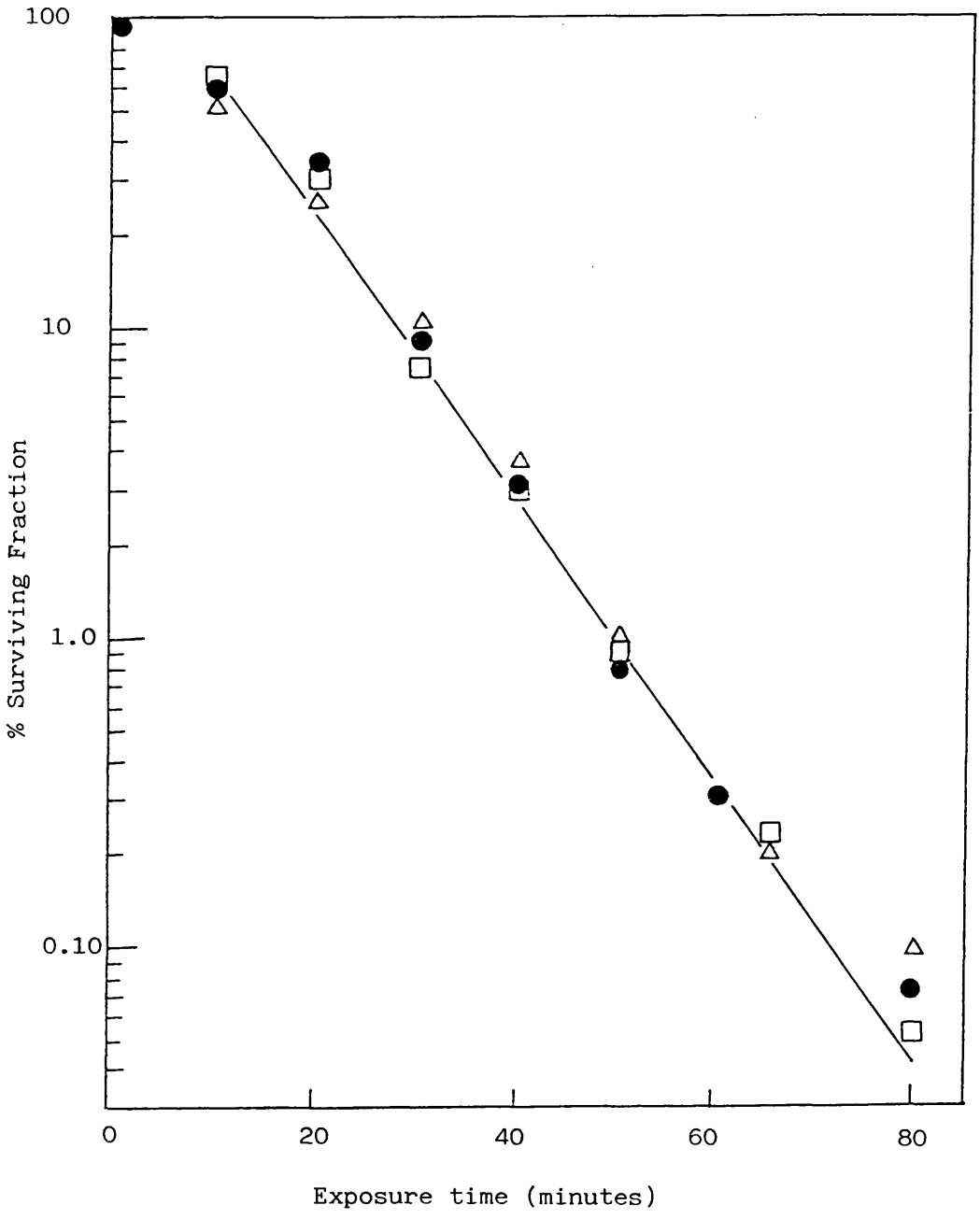


Figure 11. The inactivation by moist heat at 110°C of three replicates of spores of B. stearothermophilus NCIB 10814 (B9F) grown on solid SSMAVIT showing the survivor curve of best fit (R^2 = 0.9831)

survival data is not included in the calculations, it is possible to use linear regression analysis to give an indication of the reproducibility of the survivor curves within and between the batches. An Instat computer statistical program (Reading University) run on a BBC Master 128 Microcomputer was used to perform linear regression analysis of these results. Data displayed in Tables 11 and 12 show the linear regression analysis for the triplicate heat resistance determinations at 110°C for the two spore batches B2F and B9F respectively. These tables also show the fitted values for the survivor curves of best fit which are also displayed in Figures 10 and 11. Data for Batch B2F showed a 98.48% fit and data for batch B9F showed a 98.31% fit to a straight line model (correlation coefficient = 0.9848 and 0.9831 respectively). The results show that the method used to determine the resistance of the spores to moist heat at 110°C is reproducible and a high degree of linearity of spore survivor curves may be demonstrated.

A two sample student t-test was carried out to test the null hypothesis that there were no significant differences between the regression coefficients of the linear regression analysis displayed in Tables 11 and 12. The projected time zero (100%) survivor levels (intercepts) and the slopes of the survivor curves of best fit for triplicate determination of resistance to moist heat inactivation at 110°C were compared for the two B. stearothermophilus NCIB 10814 spore batches B2F and B9F. The hypothesis was significant at 95% confidence interval showing that there were no significant differences in the regression coefficients of the linear regression analysis of survival data from the two batches

Table 11. Data Displaying Linear Regression Analysis for Triplicate Heat Resistance Determination at 110°C of Spores of B. stearothermophilus NCIB 10814 (B2F) Grown on Solid SSMAVIT

Sample time (t) minutes	Log % surviving fraction (S/S_o) for three replicates			Regression log (% S/S_o)
	1	11	111	
10	1.7324	1.8129	1.8129	1.8343
20	1.4314	1.4771	1.5315	1.4019
30	0.9085	0.8921	0.9777	0.9696
40	0.4149	0.4771	0.6128	0.5373
50	0.1614	0.0607	0.4771	0.1049
60	-0.5229	-	-	-0.3274
65	-	-0.6989	-	-0.5436
70	-	-	-0.6021	-0.7598
80	-1.3010	-1.2218	-1.0969	-1.1921

ANOVA of Regression of log (% S/S_o) on sample time, t.

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F	R-Squared
Regression	1	20.8010	20.8010	1229.763	0.9848
Residual	19	0.3214	0.0169		
Total	20	21.1224			

Regression Coefficients

Parameter	Coefficient	SE	t-ratio
Constant (log intercept)	2.2666	0.0592	38.29
Slope	-0.0432	0.0123	-35.07

Table 12. Data Displaying Linear Regression Analysis for Triplicate Heat Resistance Determination at 110°C of Spores of B. stearothermophilus NCIB 10814 (B9F) Grown on Solid SSMAVIT

Sample time (t) minutes	Log % surviving fraction (S/S_o) for three replicates			Regression log (% S/S_o)
	1	11	111	
10	1.7782	1.7782	1.6990	1.8085
20	1.5051	1.4771	1.4150	1.3563
30	0.9031	0.8808	1.0000	0.9040
40	0.4314	0.4771	0.5563	0.4517
50	-0.2219	0.0969	0.0223	0.0005
60	-0.5229	-	-	-0.4528
65	-	-0.9586	-0.6778	-0.6789
80	-1.1549	-1.5229	-1.0458	-1.3573

ANOVA of Regression of log (% S/S_o) on sample time, t.

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F	R-Squared
Regression	1	22.2417	22.2417	1102.937	0.9831
Residual	19	0.3832	0.0202		
Total	20	22.6249			

Regression Coefficients

Parameter	Coefficient	SE	t-ratio
Constant (log intercept)	2.2608	0.0649	34.81
Slope	-0.0452	0.0014	-33.21

($t_{0.5}$, 40; d.f. = 1.68). These results show that CDM can produce bacterial spores of reproducible resistance to inactivation by moist heat at 110°C.

The Yo-values, i.e. the intercept on the y-axis of the survivor curve of best fit (9) can be obtained from the antilog of the constant parameter of the regression analysis (Tables 11 and 12) for the two batches, B2F and B9F, the Yo-values are 184% and 182.3% respectively. For both cases, the intercept ratios, IR, are greater than 1, which indicate shouldered inactivation kinetics (9).

4.4.1.1 Treatment of Survivor Curves

Linear regression analysis of survival data for bacterial spores exposed to inactivation treatments can accurately be applied to those responses that exhibit Type A survivor curve kinetics (Chapter 1, Fig. 1). In the linear regression analysis described in Section 4.4.1, time zero (100%) survival data was not included in the calculations for inactivation patterns that showed evidence of initial lag periods as in Type B survivor curves (Figs. 10 and 11). For Type D survivor curves and Type C survivor curves, the survival data for the shoulder and 'tail' phase would not be included in the analysis of regression calculations as the analysis would linearise the data to produce a log-linear survivor curve to imply Type A inactivation kinetics. The use of data derived from linear regression analysis to compare survivor curves can only be limited

to survivor curves of the same pattern. However, bacterial spores of different strains can show different survival curve patterns to similar inactivation treatments and linear regression analysis data cannot, in these cases, be used to compare resistance.

D-values have conventionally been used to compare resistances of different bacterial spore strains to similar inactivation treatments. D-values are obtained from the log-linear regions of the survivor curves and often do not take into account the initial lag periods or shoulders, activation and the tailing effect apparent in Type C survivor curves (Fig. 1). It is becoming accepted that many survivor curves obtained following inactivation of microorganisms by physical or chemical agents are sigmoidal showing an initial lag period or shoulder, a log-linear region, and a subsequent tailing. In the case of the observed linear Type A survivor curve, the initial lag or shoulder could be too short to be experimentally detected and the tailing could be apparent only at very prolonged and thus impractical exposure periods. As for Type B and D survivor curves, the shoulders are apparent but the tailing effect could be occurring at prolonged exposure periods. Type C survivor curves show the tailing with or without the log-linear region but again the shoulder region could be too short to be observed experimentally. It is therefore quite possible for different bacterial spore strains to exhibit different survivor curve types to inactivation by similar lethal treatments. Furthermore, the different survivor curve types could have equal slopes or inactivation rate constants when calculated from the

log-linear portions. It would therefore be inappropriate to use D-values to compare resistances of different bacterial spore strains to inactivation treatments when the survivor curve patterns are different. This limitation in the use of D-values to compare resistances of different bacterial spore strains to inactivation treatments may be improved by the use of the 99.9% mortality time first proposed by White (210). This is the time required to reduce the survivor level by three log cycles i.e. to 0.1% survivor level, termed here the t_3-value. The t_3-value could be considered as equivalent to an average D-value taken over three log cycles and will take into consideration the shoulder, activation or tailing often encountered in survivor curves. Therefore the use of t_3 values, while not providing a true mathematical description of the survivor curves, nevertheless seems to offer a useful practical alternative to the conventional use of D-values for comparing survivor curves. The t_3-values will be used in subsequent experiments to compare survivor curves of the selected bacterial spore strains to inactivation by similar inactivation treatments.

4.4.2 Resistance of spores of selected Bacillus strains to moist heat at 110°C

Having established the reproducibility of the method used to determine the moist heat resistance of bacterial spores produced on CDM, the next stage was to investigate the moist heat resistance of spores of each of the four strains produced on the two solid chemically defined media.

Triplicate determinations of moist heat resistance were carried out on each spore batch at 110°C, pH = 6.2. Figure 12 displays the mean survivor curves constructed from the triplicate moist heat resistance determinations for spores of B. stearothermophilus NCTC 10003 and B. subtilis Trav 5230 produced on the two CDM. The data obtained for the spores of B. stearothermophilus NCIB 8224 and NCIB 10814 produced on the two CDM are displayed in Fig. 13.

It can be seen from Figs. 12 and 13 that the four strains under investigation display a range of survivor curve types. The inactivation of spores of B. stearothermophilus NCIB 10814 produced on C-Ltd medium shows some signs of activation of dormant spores during the earlier stages of heating. This is reflected by the increase in the percentage of survivors up to 110% followed by a logarithmic death. This shape of survivor curve is often described as Type D. When produced on solid SSMAVIT medium, the strain exhibits a Type B survivor curve with no initial increase in the percentage of survivors and instead an initial lag period prior to the logarithmic decline. Spores of B. subtilis Trav 5230 produced on both CDM show Type B survivor curves. Spores of B. stearothermophilus NCIB 8224 produced on both CDM exhibit the classical Type A survivor curve with a constant fraction of the spore population being inactivated per unit time. Spores of B. stearothermophilus NCTC 10003 showed different survivor curve types depending on the CDM used. When produced on C-Ltd medium, Type A survivor curves were obtained whilst spores produced on solid SSMAVIT medium

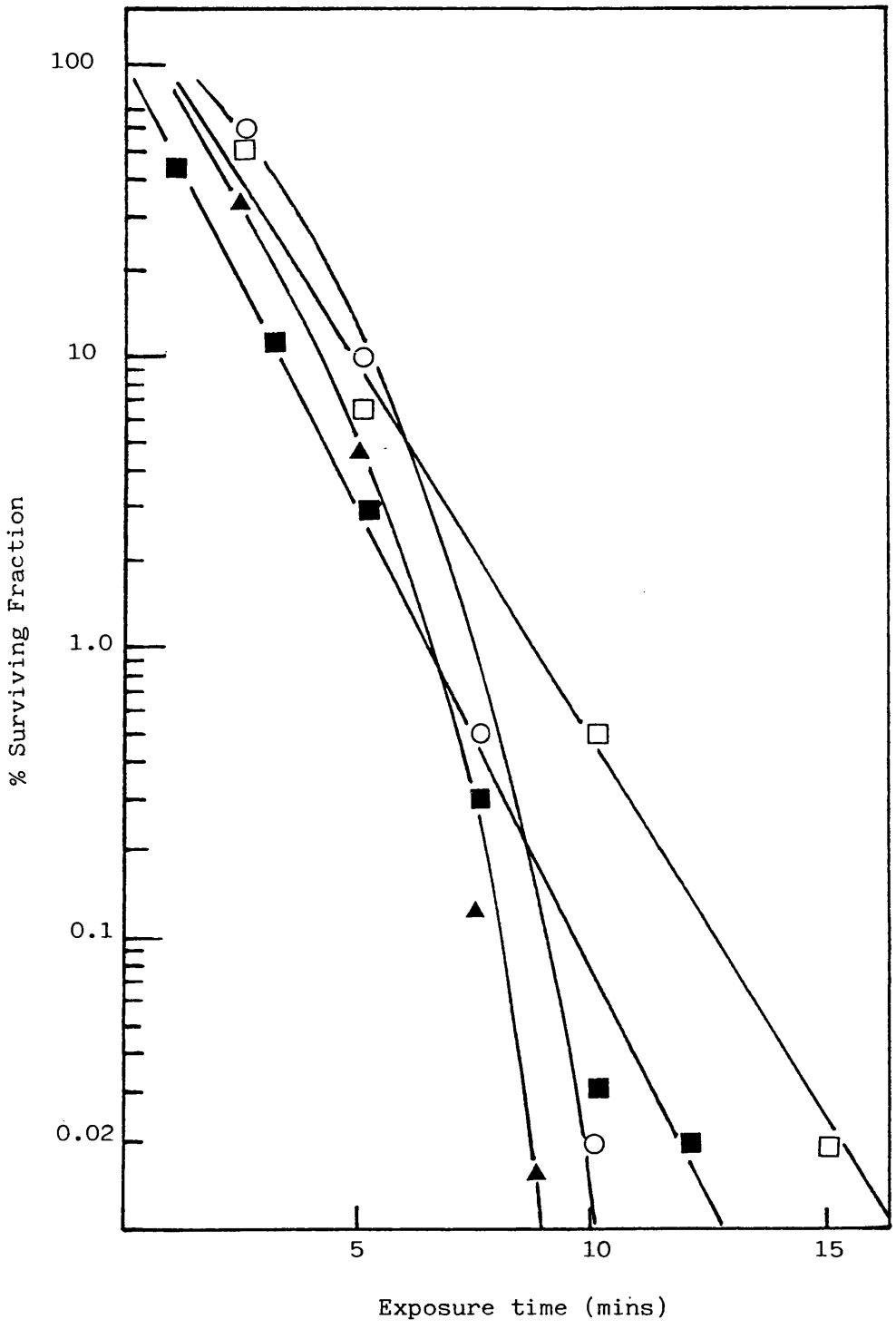


Figure 12. The inactivation of spores of Bacillus strains by moist heat at 110°C; pH = 6.2.

- □ B. stearothermophilus NCTC 10003 produced on SSMAVIT medium.
- ■ B. stearothermophilus NCTC 10003 produced on C-Ltd medium.
- ○ B. subtilis Trav 5230 produced on SSMAVIT medium
- ▲ B. subtilis Trav 5230 produced on C-Ltd medium.

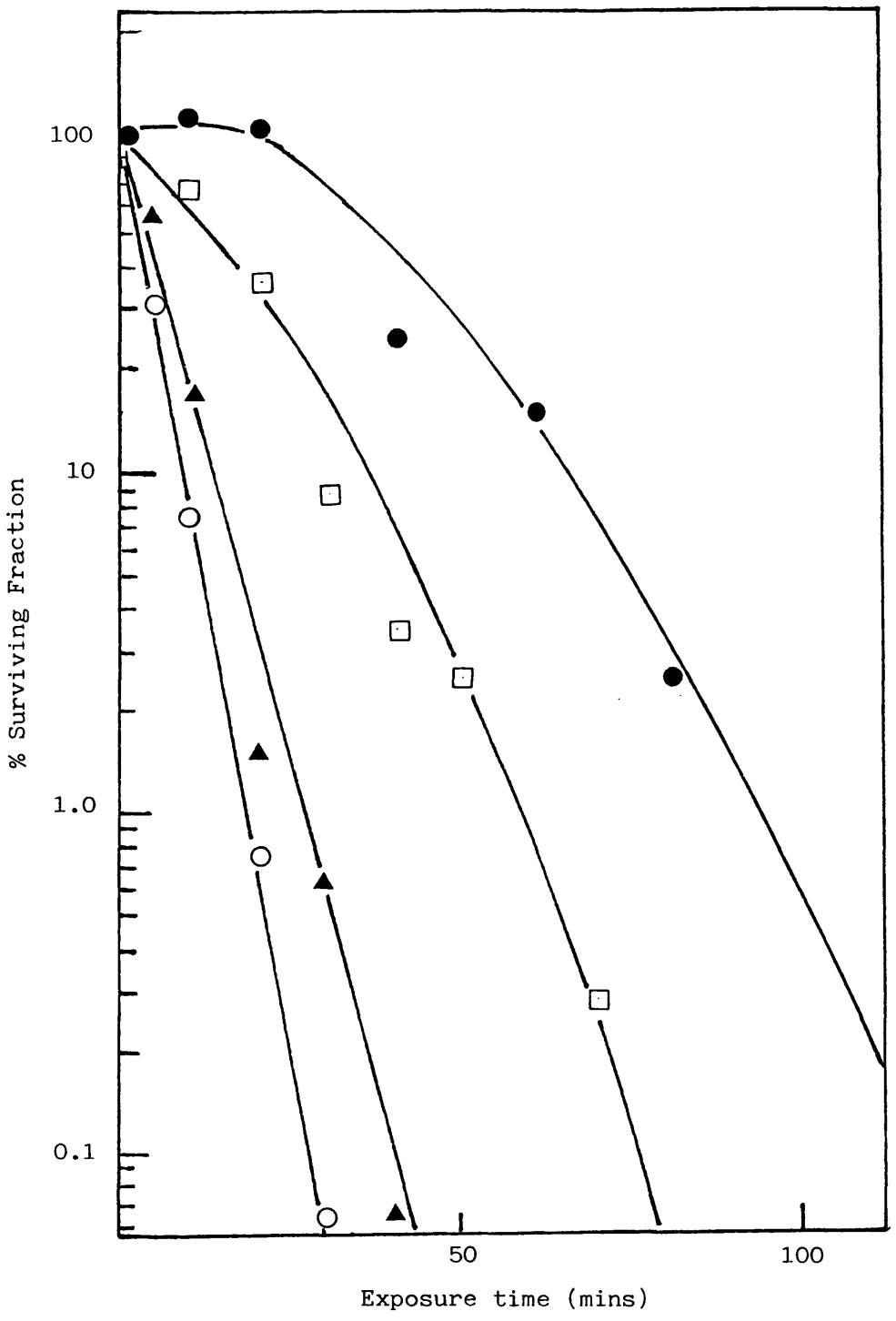


Figure 13. The inactivation of spores of Bacillus strains by moist heat at 110°C pH = 6.2.

- ● B. stearothermophilus NCIB 10814 produced on C-Ltd medium.
- □ B. stearothermophilus NCIB 10814 produced on SSMAVIT medium.
- ▲ B. stearothermophilus NCIB 8224 produced on C-Ltd medium
- ⊙ B. stearothermophilus NCIB 8224 produced on SSMAVIT medium.

produced Type B survivor curves with evidence of an initial lag period.

The variation in survivor curve type observed in these studies emphasises that the use of linear regression analysis and constants derived from it such as the inactivation rate constants k, intercept values and D-values, would be difficult to justify. For the purpose of comparing the relative resistance to moist heat at 110°C of spores of the four Bacillus strains, the t_3-values were determined from the mean survivor curves and are recorded in Table 13. These results show that spores of B. stearothermophilus NCIB 10814 produced on C-Ltd medium were the most resistant to inactivation by moist heat at 110°C (t_3 = 120 mins). Spores of B. subtilis Trav 5230, produced on SSMAVIT medium were the most sensitive to the inactivation treatment (t_3 = 7.50 mins). The resistances of the spores of the other two Bacillus strains were intermediate between those of B. stearothermophilus NCIB 10814 and B. subtilis Trav 5230 with B. stearothermophilus NCIB 8224 being more resistant (t_3 = 40 min) than spores of B. stearothermophilus NCTC 10003 (t_3 = 9.63 mins). These results also show that spores of B. stearothermophilus strains NCIB 10814, NCIB 8224 and B subtilis Trav 5230 were more resistant to the inactivation treatment when produced on C-Ltd medium than when produced on solid SSMAVIT medium. Spores of B. stearothermophilus NCTC 10003 produced on SSMAVIT medium were more resistant to the inactivation treatment than those produced on C-Ltd medium.

Table 13. Data to Summarise the Inactivation of Spores of Four Selected *Bacillus* Strains Grown on Two Solid CDM by Moist Heat at 110°C, pH = 6.2

Strain	CDM	Survivor Curve Type	t_3-value (mins)
B. stearothermophilus NCIB 10814	C-Ltd	D	120.0
B. stearothermophilus NCIB 10814	SSMAVIT	B	75.0
B. stearothermophilus NCIB 8224	C-Ltd	A	40.0
B. stearothermophilus NCIB 8224	SSMAVIT	A	28.0
B. stearothermophilus NCTC 10003	C-Ltd	A	9.53
B. stearothermophilus NCTC 10003	SSMAVIT	B	13.0
B. subtilis Trav 5230	C-Ltd	B	8.75
B. subtilis Trav 5230	SSMAVIT	B	7.50

4.5 DISCUSSION

In the previous chapter, chemically defined sporulation media have been shown to be capable of producing suspensions of spores of reproducible germination index. The data presented in Figures 10 and 11 show that growth on solid SSMAVIT medium also produces spores with reproducible moist heat resistance. These results, together with the results of the comparison of the linear regression coefficients of survival data from the inactivation of spores of B. atearothermophilus NCIB 10814 from two batches when produced on SSMAVIT medium, show that chemically defined media can produce spores with consistent characteristics.

The next stage was to compare the resistances of the spores of the selected Bacillus strains produced on the two chemically defined media to inactivation by moist heat at 110°C. Various factors are known to influence thermal resistance of bacterial spores and can be divided into pre-treatment influences, conditions prevailing during treatment and post-treatment recovery (34). For the purpose of this study, conditions during treatment and after treatment were not varied but only the composition of the sporulation media changed. The results obtained in this investigation confirm the findings of Baylis et al. (199) who showed that the composition of sporulation medium can influence bacterial spore resistance to harmful agents, in this case the harmful agent being moist heat at 110°C, pH 6.2.

The composition of the sporulation medium has been reported to influence not only spore resistance but also the shape of the survivor curves. Spores of B. megaterium produced on a calcium chloride enriched media produced shoulders on the survivor curves to inactivation by moist heat at 90°C whereas when produced on other media Type C survivor curves were obtained (72). In this study spores of B. stearothermophilus NCIB 10814 produced on SSMAVIT medium exhibited Type B survivor curves when inactivated by moist heat at 110°C. However, when produced on C-Ltd medium, Type D survivor curves were exhibited (Fig. 13). These spores exhibited a low Germination Index of 3% (Section 3.5). Their activation by moist heat at 110°C produced a Type D survivor curve which is in agreement with the report by Davies et al. (11) that spores of B. stearothermophilus with low Germination Index can display activation when exposed to heat treatments. Spores of B. stearothermophilus NCTC 10003 produced on SSMAVIT medium showed Type B survivor curves and Type A survivor curves when produced on C-Ltd medium (Fig. 12). The change in the composition of CDM did not seem to affect the survivor curve shapes for spores of B. stearothermophilus NCIB 8224 and B. subtilis Trav 5230 when inactivated by moist heat at 110°C. It is desirable for spores for use in development of biological indicators to exhibit log-linear inactivation kinetics and the choice of sporulation medium to ensure log-linear death kinetics is therefore of importance.

With the exception of spores of B. stearothermophilus NCTC 10003 whose resistance when produced on C-Ltd medium was less than

that when produced on SSMAVIT medium, the Bacillus spores tested showed greater moist heat resistance at 110°C when produced on C-Ltd medium. Table 14 shows a comparison of the constituents of solid SSMAVIT and C-Ltd media. C-Ltd medium has relatively fewer constituents which are invariably present in lower concentrations than in SSMAVIT medium. It is generally accepted that the presence of divalent cations in the sporulation medium improves thermal resistance of bacterial spores (66) with calcium and manganese ions as the most important divalent cations related to moist heat resistance (68). Spores with reduced heat resistance were produced from media that were deficient in calcium (211) and Levinson and Hyatt (72) produced spores of B. megaterium with increased heat resistance when they used a calcium chloride enriched sporulation medium. Aoki et al. (67) proposed that together these ions formed stable complexes with Dipicolinic Acid (DPA) thereby protecting the spore enzymes from inactivation. Other divalent cations such as magnesium, iron and zinc may also be involved in influencing spore resistance but there seems to be no published data to relate them directly with heat resistance of bacterial spores. The presence of suitable phosphate ion concentrations was reported to increase spore heat resistance (212) but this was later disputed by Levinson and Hyatt (72) who demonstrated reduced heat resistance in spores of B. megaterium when produced in CDM with increased phosphate concentrations.

From a comparison of the divalent cation content of the two CDM (Table 14), it would be expected that spores of higher moist

Table 14. Comparison of Compositions of the Two Chemically Defined Media

Constituent	MOLAR CONCENTRATIONS SSMAVIT	C-Ltd
D-Glucose	8.33×10^{-3}	1.0×10^{-3}
Citrate	3.20×10^{-2}	-
L-Glutamic Acid	3.02×10^{-2}	2.40×10^{-3}
L-Methionine	1.68×10^{-4}	4.50×10^{-5}
Other a. acids*	PRESENT	NONE
Fe^{2+}	1.00×10^{-5}	1.00×10^{-5}
Mg^{2+}	1.00×10^{-5}	1.80×10^{-5}
Zn^{2+}	5.00×10^{-5}	-
Ca^{2+}	1.00×10^{-3}	5.50×10^{-5}
Mn^{2+}	5.00×10^{-5}	1.50×10^{-5}
H_2PO_4	3.21×10^{-2}	1.76×10^{-2}
HPO_4	3.21×10^{-2}	1.76×10^{-2}
Cl^-	2.05×10^{-3}	8.80×10^{-5}
SO_4	6.00×10^{-5}	1.00×10^{-5}
Na^+	9.6×10^{-3}	3.52×10^{-2}
K^+	8.2×10^{-2}	7.3×10^{-3}
Thiamine HCl	4.45×10^{-7}	2.50×10^{-8}
Nicotinic Acid	1.22×10^{-5}	6.00×10^{-7}
Biotin	4.09×10^{-8}	8.00×10^{-10}
Other vitamins**	PRESENT	NONE
Tris Buffer Base	2.98×10^{-2}	-

Other a. acids*
L-leucine 9.51×10^{-4}; L-Histidine HCl 4.51×10^{-4};
tryptophan $1.22 \times 1-^{-4}$; L-Valine 7.68×10^{-4}

Other vitamins** Pyridoxal HCl 5.38×10^{-7}; Folic acid 1.36×10^{-7}

heat resistance would be produced from SSMAVIT medium, which contains higher concentrations of calcium and manganese. SSMAVIT medium also contains higher phosphate ion concentrations, C-Ltd medium is deficient in zinc but has a higher magnesium ion concentration than SSMAVIT. Higher resistance to moist heat at 110°C by spores produced on SSMAVIT medium was only demonstrated by spores of B. stearothermophilus NCTC 10003. Other factors must be involved since higher cation content was not observed in practice to increase the resistance of the spores of the other selected Bacillus strains to inactivation by moist heat at 110°C. It is therefore not always possible to relate heat resistance to specific nutrients.

Different sporulation media may produce spores of different physical and chemical structure which show different responses to inactivation by heat and chemicals (60, 199). It is possible that C-Ltd medium might be producing spores with stronger heat protective structures despite this medium's deficiency in divalent cation content.

Spores of Bacillus species are unusual in the very wide range of resistances they exhibit to the same inactivation treatment. This is exemplified by the diversity of the t_3-values obtained for the selected strains in this study (Table 13). These major differences in heat resistance shown by the spores, irrespective of the sporulation media, are due to genetic variation between spores of different species and also between strains of the

same species (34). Warth (46) reported that differences in heat resistance between species are dependent not only on effectiveness of the heat resistance mechanism of the spore, but also to a large degree upon the temperature to which the species is adapted. The results of this investigation confirm that thermophilic strains of Bacillus species have thermoresistance generally higher than that of mesophiles or psychrotrophs (34, 46) probably due to presence of more heat-stabile enzymes in the thermophiles. This is exemplified by the differences between the higher t_3-values for the B. stearothermophilus strains and the lower t_3-values for B. subtilis Trav 5230, the mesophile.

It was found difficult to compare the resistance of the spores of the selected Bacillus strains to inactivation by moist heat at 110°C with published bacterial thermal resistance data because different workers often use different methods to determine spore resistance. The influence of the composition of sporulation medium on resistance of spores has been discussed and authors often use different CDM. The suspending medium used in heat resistance experiments can also influence bacterial spore resistance (34). Recovery conditions of the treated spores can also influence resistance data. While it is not advisable to standardise methods of determining moist heat resistance of bacterial spores there is however need for a realistic approach in reporting thermal resistance data particularly in situations where the survivor curves are not log-linear over the entire exposure period. Pflug et al. (20) proposed the use of the intercept ratios (IR) to

differentiate between different survivor curve shapes. Heat resistance is conventionally expressed in terms of D-values and the limitation in the use of D-values has been encountered in this study. Furthermore, D-values are often cited without indication of recovery conditions, survivor curve shapes and sporulation media. The use of t_3-values has been recommended in this study to express bacterial spore resistance. By definition, the t_3-value is equal to 3 times the D-value for log-linear inactivation kinetics. On this basis, t_3-values obtained in this study were compared with some of the data cited by different workers, assuming of course that the D-values were obtained from log-linear survivor curves.

Spores of B. stearothermophilus NCIB 8224 produced on solid SSMAVIT had a t_3 value of 28 minutes which is comparable to 3 times the D-value of 8 minutes reported by Hoxey (22) under similar test conditions. Thorpe (213) reported a D-value of 5.24 mins (i.e. t_3 = 15.72 mins) for the inactivation of spores of B. stearothermophilus NCIB 8224 inactivated by moist heat at 115.6°C. The t_3 value is considerably lower than the one reported in this study but then the inactivation experiment was carried out at a higher temperature, a different sporulation and recovery media were used and finally the spores were suspended in McIlvaine's buffer solution of pH 6.0. Spores of B. subtilis Trav 5230 produced on SSMAVIT medium showed a t_3-value of 7.5 minutes which is also comparable to the value of 6 minutes derived from the D-value of 2 min reported by Hoxey (22). Anderson et al. (66) reported D-values between 50-92 minutes for the inactivation of spores of B.

stearothermophilus NCTC 10003 by moist heat at 110°C (i.e. t_3 = 150 - 276 mins). These values are more than 10 times the t_3-values obtained for the same strain in the investigation reported in this study, but then the determination by these workers was carried out in acetate buffer of pH 5 and different chemically defined sporulation media were used at sporulation temperatures between 50°C and 70°C. In this study, spores were suspended in unbuffered sterile glass-distilled water of pH 6.2.

The data presented in this chapter show a wide range of resistance between the spores of the selected Bacillus strains and together with the data presented in Chapter 3, suggest that it might not be easy to produce spores displaying properties of an ideal biological indicator in high germination index, high heat resistance, high yield and log-linear inactivation kinetics. While these characteristics might have been examined separately, the factors influencing these properties will affect the choice of a suitable strain for development to a biological indicator organism. These characteristics might have to be compromised to get the best candidate spore. It was observed in the previous chapter that C-Ltd medium produced high spore yields (90%) of B. stearothermophilus NCIB 8224 and NCTC 10003. These spores were of desirable high germination index, 68% and 78% respectively and required minimal incubation periods for sporulation. A high thermal resistance with log-linear inactivation kinetics added to the above characteristics, would produce a potential biological indicator organism. B. stearothermophilus NCIB 10814, produced on C-Ltd

medium was the most resistant to inactivation by moist heat at 110°C, pH 6.2 (t_3 = 120 min). However, spores of this strain exhibited a type D survivor curve and their low GI value (3%) makes it unsuitable for development to a biological indicator. At the other end of the sensitivity spectrum, spores of B. subtilis Trav 5230, which have been reported to have a tendency for shouldered inactivation kinetics (22) were considered too sensitive. Spores of B. stearothermophilus NCTC 10003 showed resistance intermediate between that of the spores of the thermophiles B. stearothermophilus NCIB 10814 and NCTB 8224 and the mesophile, B. subtilis Trav 5230 but exhibits shouldered survivor curves. By a process of elimination spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium would appear to be the most suitable, of the organisms tested, for further development as a biological indicator organism for LTSF sterilization.

CHAPTER 5

EFFECTS OF TEMPERATURE AND STORAGE ON THE RESISTANCE OF SPORES OF SELECTED *BACILLUS* SPECIES TO INACTIVATION BY FORMALDEHYDE IN AQUEOUS SOLUTION

5.1 INTRODUCTION

Experiments reported in the previous chapter were performed to determine the resistance of spores of the selected Bacillus strains to inactivation by moist heat at 110°C. On the basis of this criterion and other characteristics described in Chapter 2, B. stearothermophilus NCIB 8224 produced on C-Ltd medium was selected as the best candidate for development as a potential biological indicator organism for LTSF sterilization.

Biological indicators to be used to monitor LTSF processes will be exposed to the extra insult of the bactericidal chemical, formaldehyde. It is understood that spore resistance to one lethal agent is not necessarily related to the resistance to other inactivating agents (199). It is desirable for the selected spores for development as biological indicator organsims to possess a high resistance to each parameter of the LTSF process in isolation. Experiments described in this chapter were carried out to determine the resistance of spores of each of the selected Bacillus strains produced on chemically defined media to inactivation by formaldehyde in aqueous solution.

The effects of the temperature of inactivation and cold room storage on the resistance of the spores to inactivation by formaldehyde in aqueous solution were also investigated.

5.2 METHODS

5.2.1 Preparation and Standardization of Aqueous Formaldehyde Solutions

All the investigations on the resistance of the selected bacterial spores to inactivation by formaldehyde in aqueous solution were carried out in a 0.5% w/v solution. This strength was chosen as it is equivalent to the upper formaldehyde concentration limit currently used in LTSF sterilizers (7, 74). This concentration would also ensure the predominance of the hydrated formaldehyde monomer (methylene glycol) and the unhydrated formaldehyde monomer with little traces of low molecular weight polymeric forms at temperatures up to 100°C (109). Low aqueous formaldehyde concentrations were reported by Walker (109) to favour the presence of methylene glycol and at concentrations up to 2%, dissolved formaldehyde is principally monohydrate with small concentrations of unhydrated formaldehye monomers (109). It is this monomeric form upon which the sporicidal activity of formaldehyde is thought to depend (123, 133). Hoxey (22) demonstrated that it was also the concentration beyond which any further concentration increases did not result in corresponding increases in the inactivation rate of spores of B. subtilis Trav 5230 at 35°C.

The formaldehyde solution was prepared by diluting 1.316 ml of the 38% w/v formaldehyde stock solution (containing 10% w/v methanol as stabilizer) (Fisons Ltd.) with sterile glass distilled

water to 100 ml. Methanol, added to prevent polymerisation to polyoxymethylene glycols, would be present in the prepared solution at 0.13% w/v concentrations. Such concentrations of methanol were demonstrated to have no significant effect on viability of bacterial spores or on the resistance of spores of B. subtilis NCIB 3610 to inactivation by moist heat at 65°C (74).

Once prepared, the 0.5% w/v aqueous formaldehyde solution was left to equilibrate to the required temperature in a glass stoppered 100 ml Grade A volumetric flask (Quickfit, Fisons Ltd.), for 12 hours in a water bath.

5.2.2 Assay of Formaldehyde Solutions

The sodium sulphite titration method (109) was used to check the concentration of the prepared formaldehyde solutions. The method is based on the quantitative liberation of sodium hydroxide when formaldehyde reacts with sodium sulphite to form a formaldehyde-bisulphite addition product.

252 g of AR grade hydrous sodium sulphite (BDH Ltd.) were dissolved and made up to 1 litre in glass distilled water. 50 ml of this molar sodium sulphite solution were transferred to a 100 ml Erlenmeyer flask and three drops of thymolphthalein indicator solution added (0.1% w/v in ethanol). The contents were carefully neutralised with 2-3 drops of normal hydrochloric acid until discolouration. An accurately measured volume of the aqueous

formaldehyde solution was then added and the resulting mixture titrated slowly with normal hydrochloric acid to complete discolouration. In this assay, 1 ml of normal hydrochloric acid would be equivalent to 0.03003 g of formaldehyde. This method detects all the dissolved formaldehyde but does not distinguish between monomeric and polymeric forms. The concentration of formaldehyde in the prepared solutions varied by 0.0001% which was considered insignificant.

5.2.3 Inactivation of Formaldehyde

Apart from its sporicidal activity, formaldehyde can also inhibit bacterial spore germination (123). It is therefore necessary to prevent the carry over of inhibitory formaldehyde concentrations to the recovery medium. Various methods that have been reported to inactivate formaldehyde solutions have been reviewed in Chapter 1.

For the purpose of this investigation, 10% w/v glycine solution (BDH Ltd.) was used to inactivate 0.5% w/v formaldehyde. In solution, formaldehyde readily combines with glycine (amino-acetic acid) in equimolar proportion to give methylene-aminoacetic acid (109). The excess glycine in this case would guarantee complete and precise inactivation of formaldehyde. To minimise the introduction of inactivator traces to the recovery medium, the final dilution was filtered and the membrane filter washed three times by passing 2 ml volumes of sterile glass

distilled water through it. The filter was then incubated on the recovery medium at the appropriate temperature to allow the growth of surviving spores.

To demonstrate the reproducibility of this technique, a quintuplicate determination of the viable count of spores of B. stearothermophilus NCIB 8224 batch (B6F) was carried out using sterile 10% w/v glycine as diluent. 1 ml aliquots of the final dilutions were filtered through 0.45 µm, 47 mm diameter membranes in a negative pressure filtration apparatus (Sartorius Ltd.). The washed filters were incubated on overdried Nutrient Agar plates and incubated at 56°C for five days. Further incubation up to 10 days did not increase recovery. Data in Table 15 show the results of the quintuplicate determination of viability of spores of B. stearothermophilus NCIB 8224 batch (B6F) and the analysis of variance of the data.

The analysis of variance confirms that variation between samples is not greater than within replicates from a single sample and that the overall coefficient of variation (3.5%) is within statistically acceptable limits.

5.2.4 Method for the Determination of Resistance of Spores of Selected Bacillus Strains to inactivation by 0.5% w/v Aqueous Formaldehyde Solution

A suitable amount of the equilibrated 0.5% w/v formaldehyde

Table 15. Quintuplicate Determination of the Viable Count of Spores of B. stearothermophilus NCIB 8224 (B6F) recovered on membrane-filters incubated on NA at 56°C using 10% w/v glycine as diluent and the Analysis of Variance of the Data

Sample	Dilution Factor	Colony Counts	Mean Colony Count	Mean Viable Count/ml
1		65,63,62,67,68		
2	1×10^6	63,59,67,65,64	64.68	6.47×10^7
3		60,67,64,65,68		
4		67,64,64,69,67		
5		65,62,63,64,65		

Analysis of Variance

Source of Variance	Degrees of Freedom	Sum of Squares	mean Square	F
Between samples	4	21.84	5.46	1.0
Within samples	4	42.24	10.56	2.0
Residual	16	83.36	5.21	
Total	24	147.44		

Overall Coefficient of Variation (C.V.) = 3.5%

F (4, 16) $P_{0.05}$ = 3.01

solution was transferred to a sterile 100 ml glass vessel incorporating two sampling ports. The vessel was fitted with a condenser unit (Quickfit; Fisons ltd.) to avoid any loss of formaldehyde by evaporation. The assembly was secured with the vessel fully immersed in a water bath set at the desired temperature. The water bath (Grant Instruments) maintained the temperature with a 0.01% variation. An appropriate volume of spore suspension was added through one of the sampling ports to give an initial concentration of approximately 5×10^6 spores/ml. A parallel dilution was performed in sterile glass distilled water to enable the initial viable count of untreated spore suspension at time zero (100% survival) to be determined. A submerged magnetic stirrer (Rank Brothers Ltd.) was used to stir the inactivation mixture at 90 rpm. Samples (1 ml) were removed through the sampling port at predetermined time intervals and aseptically added to 9 ml of sterile 10% w/v glycine solution to inactivate the residual formaldehyde. After two minutes contact, the solutions were serially diluted in sterile glass distilled water (1.0 ml + 9.0 ml). 2 ml triplicate volumes of the final dilutions were filtered through 47 mm, 0.45 µm pore size cellulose nitrate filters (gridded) in a negative pressure filtration apparatus (Sartorius Ltd.). Each of the three membrane filters was washed three times by passing 2 ml volumes of sterile water through them. The membrane filters were then aseptically transferred to the surface of overdried recovery medium. Plates were incubated inverted at 56°C (37°C for B. subtilis Trav 5230) for 5 days. Survivors were then counted and survivor curves constructed.

5.3 EXPERIMENTAL

5.3.1 Reproducibility of Bacterial Spore Resistance to Inactivation by 0.5% w/v Formaldehyde in Aqueous Solution at 70°C

Before further investigations into the resistance of the spores to formaldehyde in aqueous solution at various temperatures could be undertaken, it was necessary to establish the reproducibility of the experimental technique to be used.

Spores of B. stearothermophilus NCIB 10814 (B2F) grown on solid SSMAVIT medium were exposed to 0.5% w/v aqueous formaldehyde solution at 70°C using the method outlined in Section 5.2.4. Figure 14 shows the mean survivor curve of triplicate determinations of resistance of the test strain to inactivation by 0.5% w/v formaldehyde at 70°C. The error bars are indicative of the standard deviations of the surviving fractions from the mean value around each sampling time. Figure 15 shows the variation in response to the same test conditions observed with spores of B. stearothermophilus NCIB 10814 from three separate batches (B2F, B3F, B9F) produced on solid SSMAVIT media. The results show that the variation in response within a single batch (Fig. 14) is comparable to that within separate batches (Fig. 15).

The experimental techniques used can be considered accurate and reproducible in determining the response of bacterial spores to

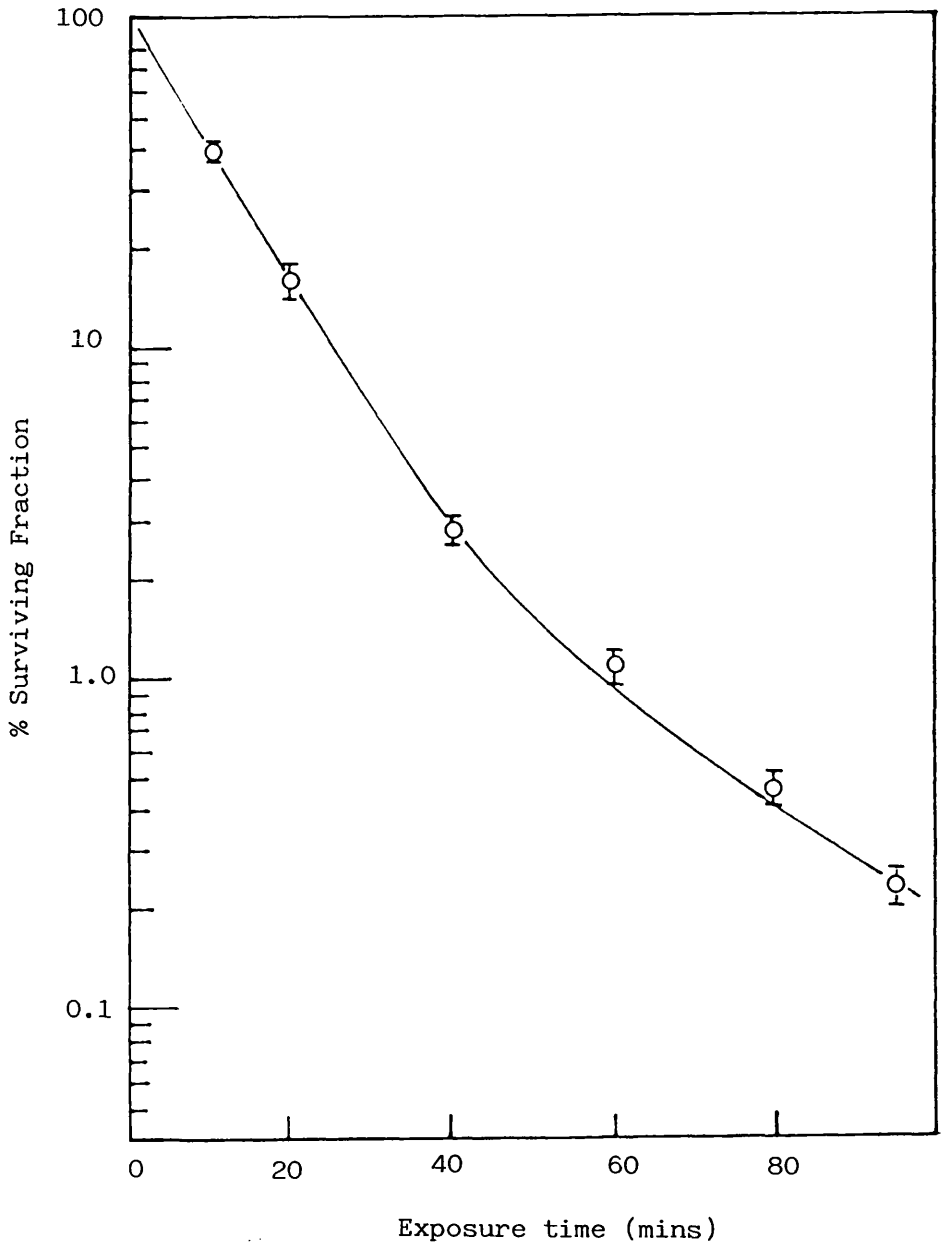


Figure 14. Variation in resistance to 0.5% w/v formaldehyde in aqueous solution at 70°C within 3 replicates of a single batch of spores of B. stearothermophilus NCIB 10814 produced on solid SSMAVIT medium.

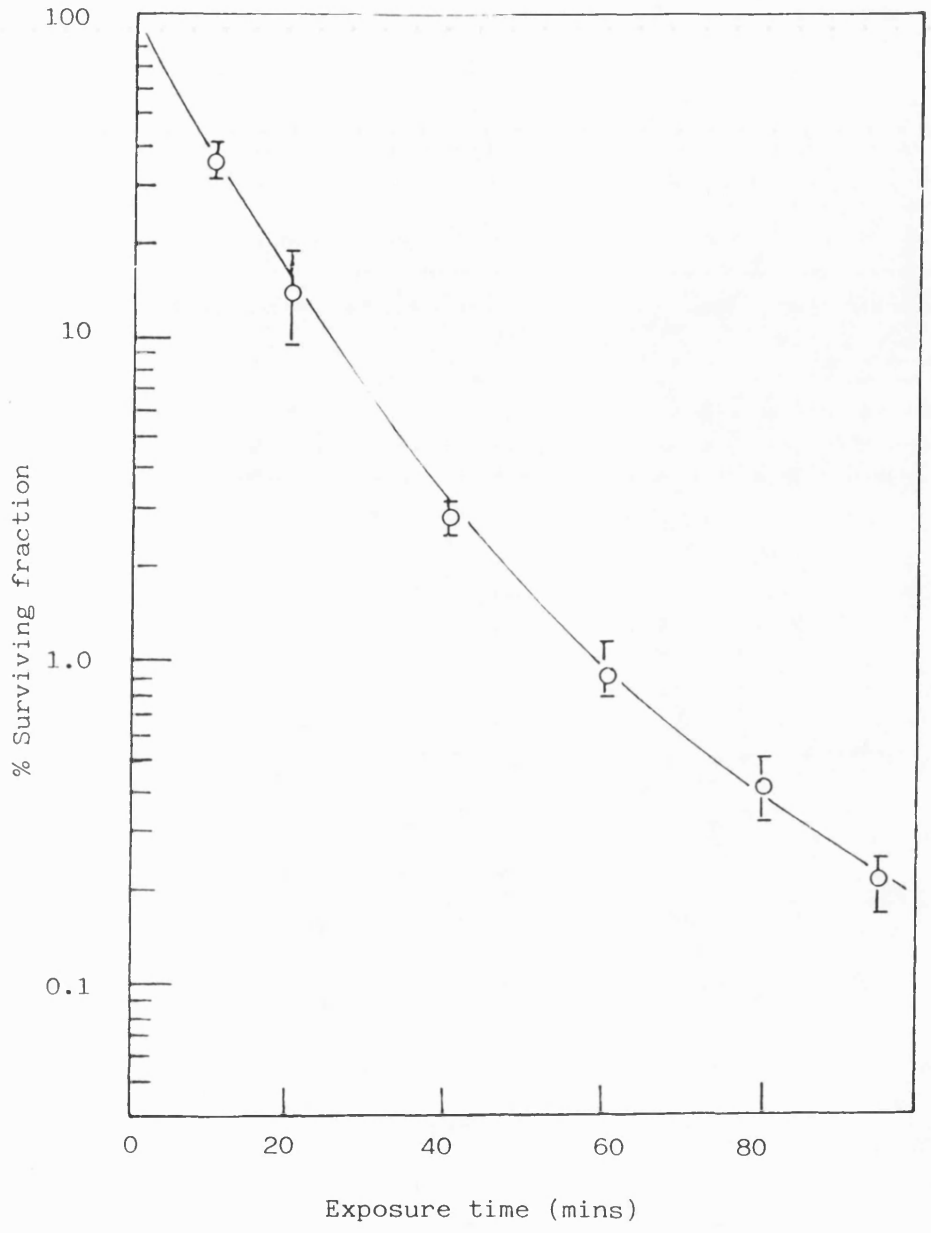


Figure 15. Variation in resistance to 0.5% w/v formaldehyde in aqueous solution at 70°C between separate batches of spores of B. stearothermophilus NCIB 10814 produced on solid SSMAVIT medium.

inactivation by 0.5% w/v formaldehyde in aqueous solution. The results also confirm that bacterial spores produced on chemically defined sporulation media are capable of exhibiting reproducible characteristics.

5.3.2 Resistance of Spores of Selected *Bacillus stearothermophilus* Strains to Inactivation by 0.5% w/v formaldehyde in Aqueous Solution at 70°C

In the U.K., the recommended temperature for LTSF sterilization is 73° ± 2°C (2, 129, 133). Recently, Alder (133) emphasised the need for much lower temperatures e.g. 55°C for the more delicate electronic equipment. Most LTSF cycles in Europe use temperatures between 65° and 80° C. A convenient temperature of 70°C was chosen to determine the resistance of spores of the selected *Bacillus* species to inactivation by 0.5% w/v formaldehyde in aqueous solution using the method described in Section 5.2.4. Survivor curves following the inactivation of the bacterial spores by 0.5% w/v aqueous formaldehyde solution were constructed as described in Chapter 1 and are displayed in Fig. 16. The 99.9% mortality values (t_3-values) in minutes, were read off these survivor curves and displayed in the order of increasing resistance in Table 16.

The data displayed in Table 16 and the survivor curves shown in Fig. 16 show a wide range of sensitivities displayed by the spores of the selected *Bacillus* species to the test conditions. As

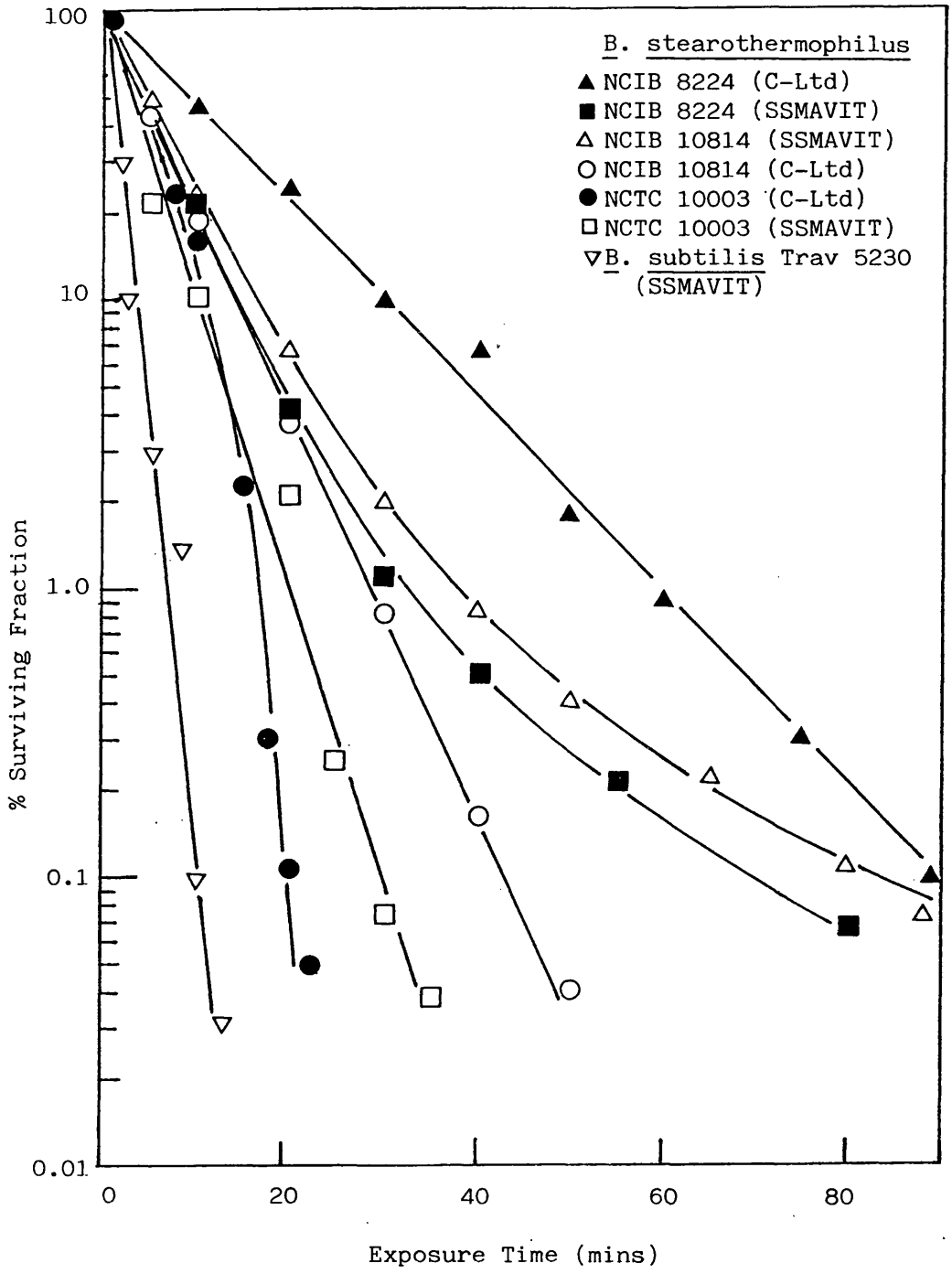


Figure 16. Inactivation of spores of Bacillus strains produced on SSMAVIT and C-Ltd media by 0.5% w/v formaldehyde in aqueous solution at 70°C

Table 16. Data showing the t_3-values (mins) and Survivor Curve Type for the Inactivation of Spores of Selected *Bacillus* Species by 0.5% w/v formaldehyde in aqueous solution at 70°C

Strain	Sporulation Medium	Survivor Curve Type	t_3 (mins)
B. subtilis Trav 5230	Solid SSMAVIT	A	10
B. stearothermophilus NCTC 10003	C-Ltd	B	20
B. stearothermophilus NCTC 10003	Solid SSMAVIT	A	30
B. stearothermophilus NCIB 10814	C-Ltd	A	43
B. stearothermophilus NCIB 8224	Solid SSMAVIT	C	68
B. stearothermophilus NCIB 10814	Solid SSMAVIT	C	84
B. stearothermophilus NCIB 8224	C-Ltd	A	90

would be expected, spores of B. subtilis Trav 5230 produced on solid SSMAVIT medium were the most sensitive (t_3 = 10.5 minutes) and displayed a log-linear Type A survivor curve. When produced on C-Ltd medium, spores of B. subtilis Trav 5230 were too sensitive to the test conditions for accurate sampling using the prescribed method and were not investigated further. The thermophilic strains were more resistant with B. stearothermophilus NCIB 8224 spores produced on C-Ltd medium displaying the highest resistance to the test conditions (t_3 = 90 minutes). This strain also exhibited a linear logarithmic death curve, Type A, over the entire exposure period. Spores of B. stearothermophilus NCTC 10003, produced on C-Ltd medium were the most sensitive of the thermophilic group exhibiting a Type B survivor curve, and a t_3-value of 20 minutes. When produced on solid SSMAVIT medium, the shape of the survivor curve changed to Type A for the entire exposure period. The t_3-value this time was 29.3 minutes showing that spores of B. stearothermophilus NCTC 10003 produced on solid SSMAVIT medium were more resistant to inactivation by 0.5% w/v formaldehyde in aqueous solution than when produced on C-Ltd medium. Spores of B. stearothermophilus NCIB 10814, produced on both solid SSMAVIT and C-Ltd medium showed resistance to the test conditions intermediate between that of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium and that of spores of B. stearothermophilus NCTC 10003. Spores of B. stearothermophilus NCIB 10814 produced on solid SSMAVIT medium were more resistant (t_3 = 8.4 min) and exhibited a Type C survival curve. When produced on C-Ltd medium, this strain showed a Type A survivor curve but was more sensitive to the test

conditions (t_3 = 43 minutes). Spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium were the most resistant (t_3 = 90 mins) and exhibited the desirable log-linear survivor curve. When produced on solid SSMAVIT medium, the spores of B. stearothermophilus NCIB 8224 were less resistant (t_3 = 68 mins) and exhibited a Type C survivor curve.

5.3.3 Effect of Temperature on the Resistance of Spores of the Three Selected Bacillus stearothermophilus Strains to Inactivation by 0.5% w/v Formaldehyde in Aqueous Solution

In the light of the various temperatures that have been reported for use in LTSF cycles (2, 129, 133, 165), it was considered necessary to determine spore resistance to inactivation by 0.5% w/v aqueous formaldehyde solution at temperatures between 60°C or 65°C and 80°C. Spores of B. stearothermophilus strains NCTC 10003, NCIB 8224 and NCIB 10814 produced on both solid SSMAVIT and C-Ltd media were used in this investigation.

Spores of each of these selected strains were exposed to temperatures of 60°C or 65°C, 67.5C°, 70°C, 72.5°C, 75°C, 77.5°C and 80°C suspended in 0.5% w/v aqueous formaldehyde solution and their resistance determined using the method outlined in Section 5.2.4. The data obtained were plotted as survivor curves of percent survivors on a logarithmic scale against exposure times on a linear scale and are displayed in Figures 17-23. Each survivor curve displayed was obtained from at least three replicate determinations

with the results pooled and the best survivor curve constructed.

The survivor curves obtained for the inactivation of spores of B. stearothermophilus NCTC 10003 produced on C-Ltd medium by 0.5% w/v aqueous formaldehyde solution at temperatures between 60° and 67.5°C were log-linear over the exposure period (Fig. 17). There was however evidence of a shoulder in the survivor curve obtained at 70°C (Fig. 17) and at temperatures to 80°C (Fig. 18). When produced on solid SSMAVIT medium, the survivor curves were initially Type C at lower temperatures (65° and 67.5°C) turning to log-linear Type A at the higher inactivation temperatures between 70° and 80°C (Fig. 19). Spores of B. stearothermophilus NCIB 10814, produced on solid SSMAVIT medium, exhibited Type C survivor curves over the entire range of temperatures tested (Fig. 20). When produced on C-Ltd medium, the survivor curves were a mixture of Type C at temperatures between 65° and 67.5°C and Type A at temperatures above 70°C (Fig. 21). Inactivation of spores of B. stearothermophilus NCIB 8224 produced on solid SSMAVIT medium produced survivor curves which revealed evidence of tailing (Type C) at temperatures between 65° and 80°C. Inactivation at 60°C produced a Type B survivor curve (Fig. 22). When produced on C-Ltd medium, this strain produced log-linear survivor curves for the inactivation at temperatures between 65° and 80°C (Fig. 23).

The spores of the Bacillus species tested in this investigation displayed a variety of survivor curve types. It has been suggested by Davies (7) that survivor curves obtained for the

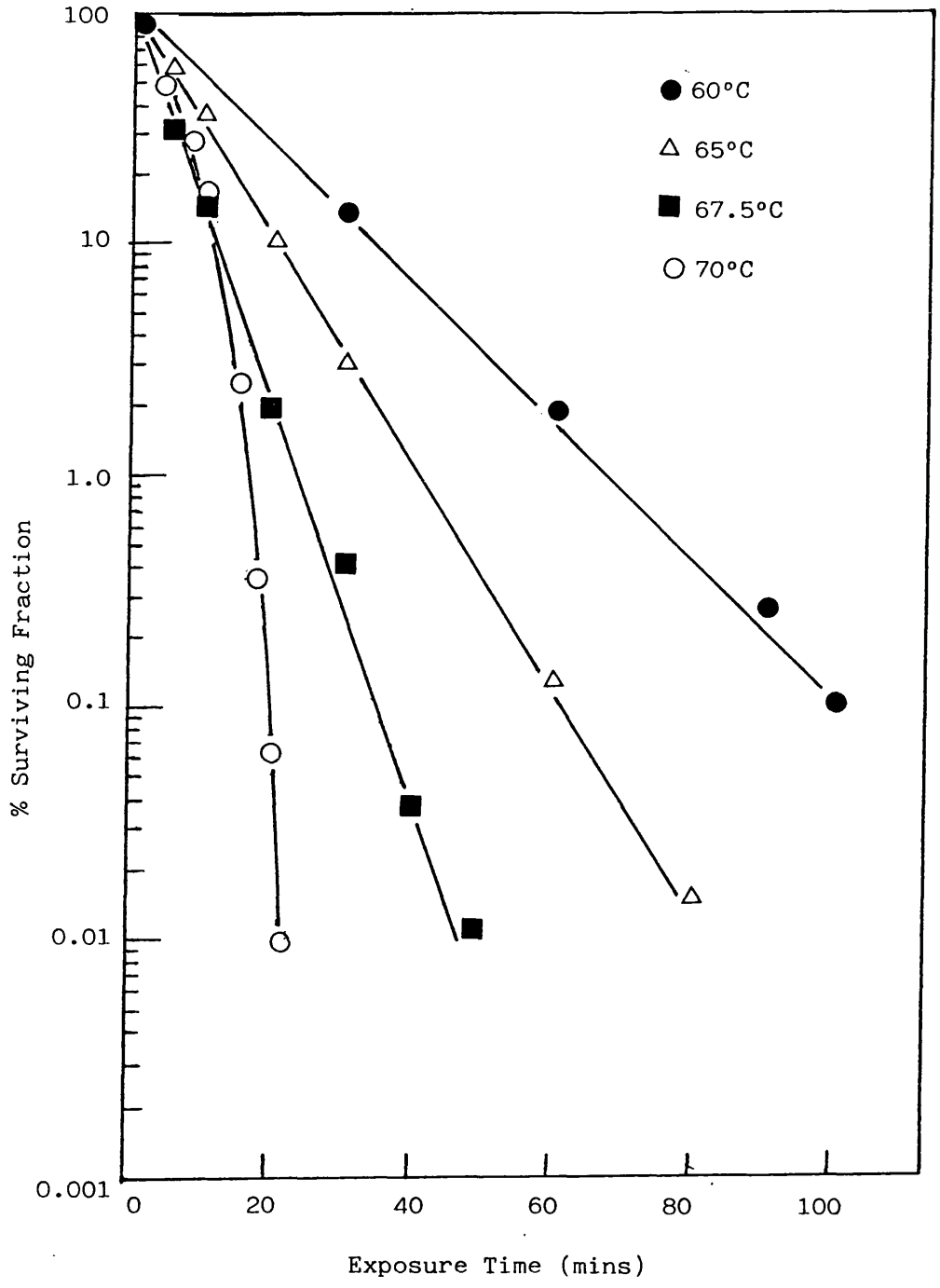


Figure 17. Effect of temperature on the inactivation of spores of B. stearothermophilus NCTC 10003 produced on C-Ltd medium by 0.5% formaldehyde in aqueous solution.

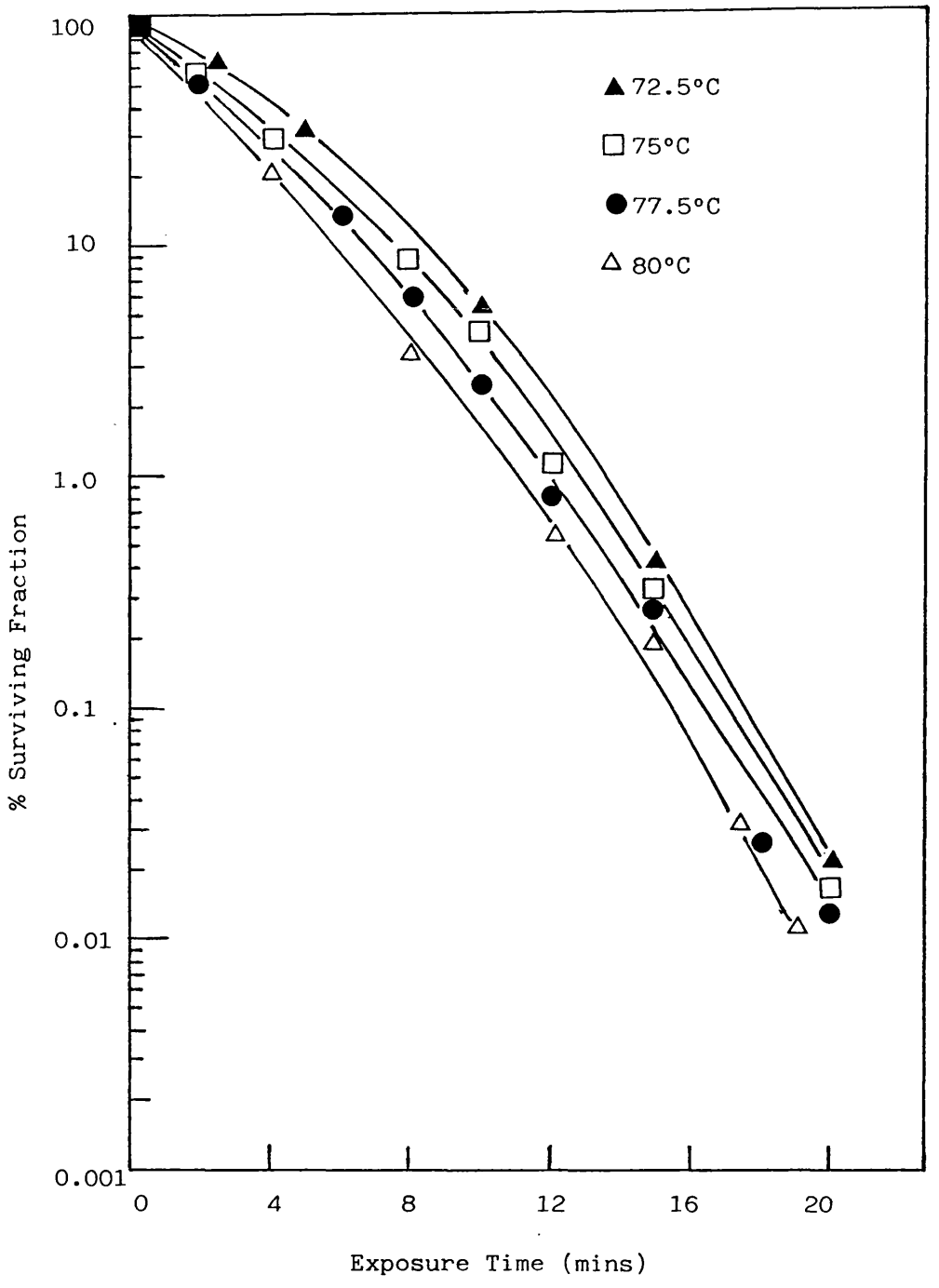


Figure 18. Effect of temperature on the inactivation of spores of B. stearothermophilus NCTC 10003 produced on C-Ltd medium by 0.5% w/v formaldehyde in aqueous solution.

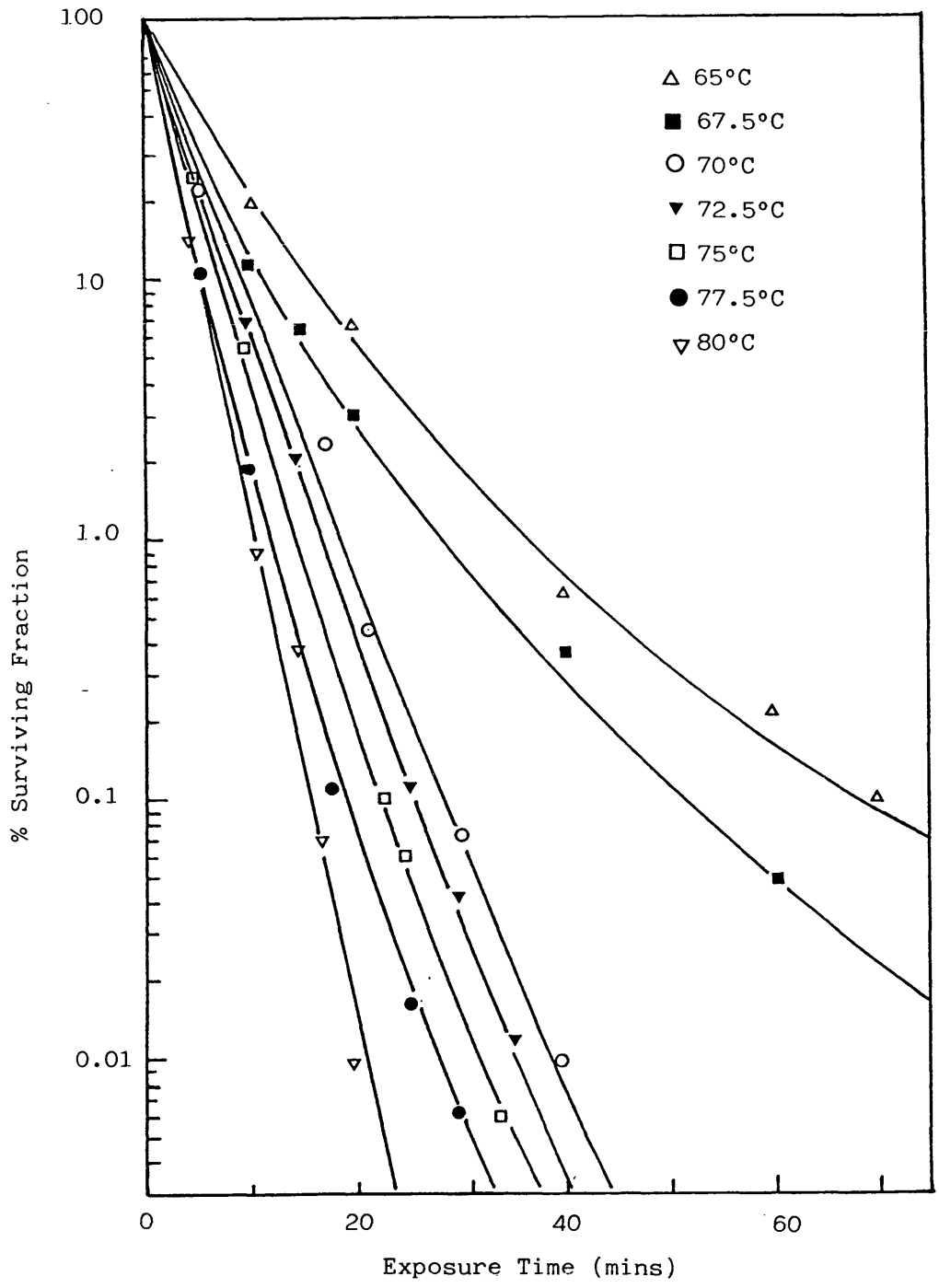


Figure 19. Effect of temperature on the inactivation of spores of B. stearothermophilus NCTC 10003 produced on SSMAVIT medium by 0.5% w/v formaldehyde in aqueous solution.

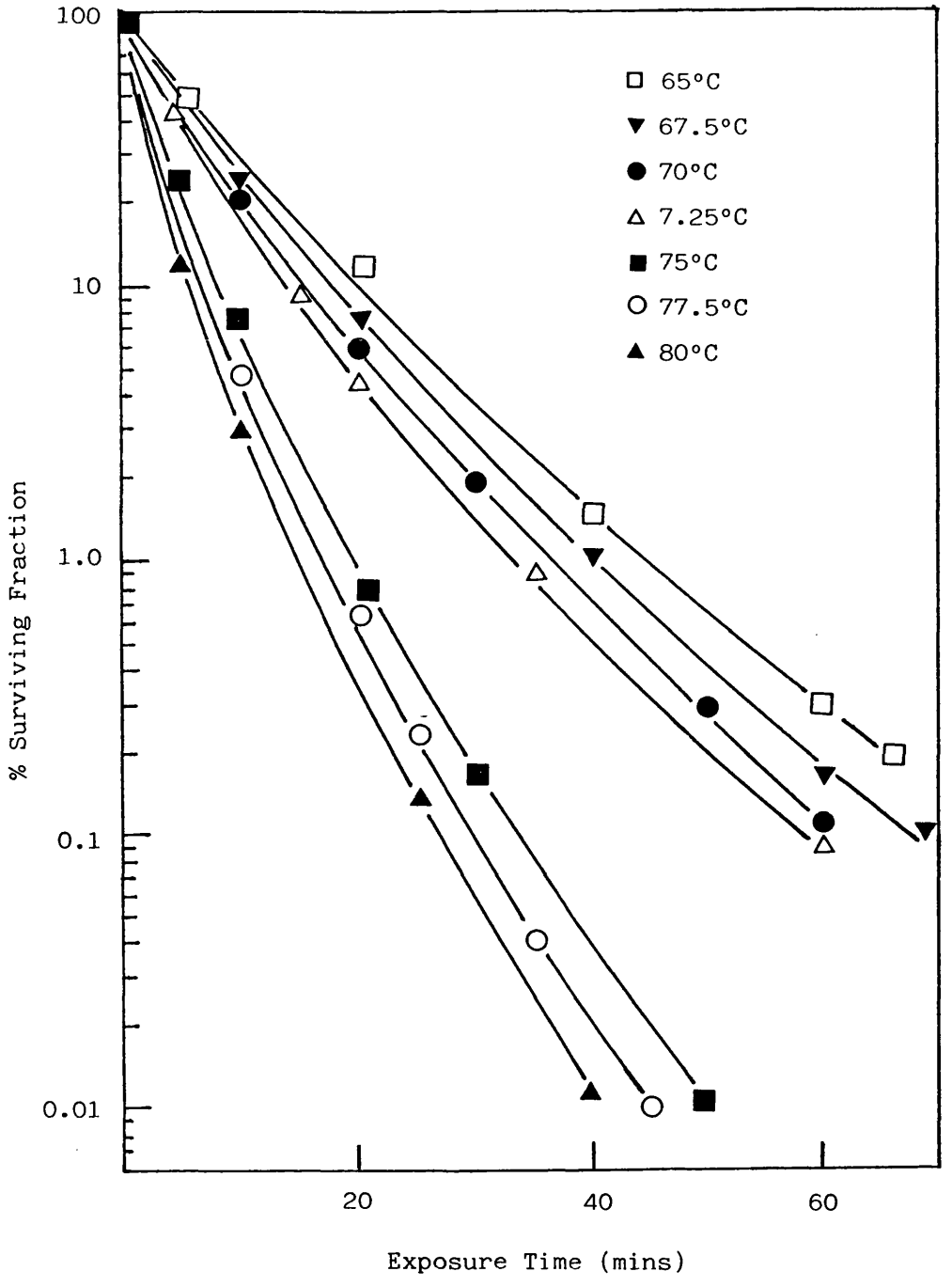


Figure 20. Effect of temperature on the inactivation of spores of B stearothermophilus NCIB 10814 produced on SSMAVIT medium by 0.5% w/v formaldehyde in aqueous solution.

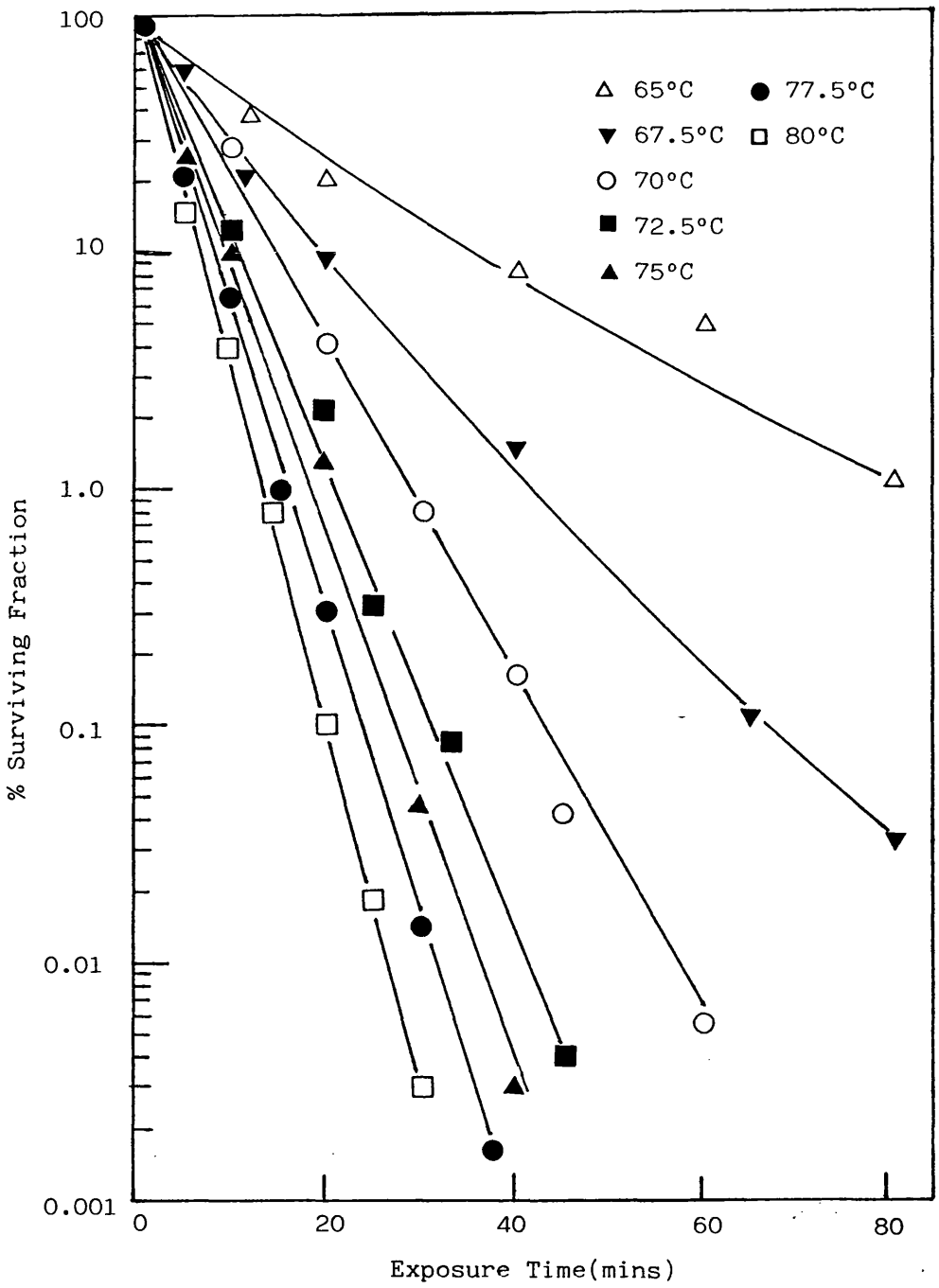


Figure 21. Effect of temperature on the inactivation o spores of B. stearothermophilus NCIB 10814 produced on C-Ltd medium by 0.5% w/v formaldehyde in aqueous solution.

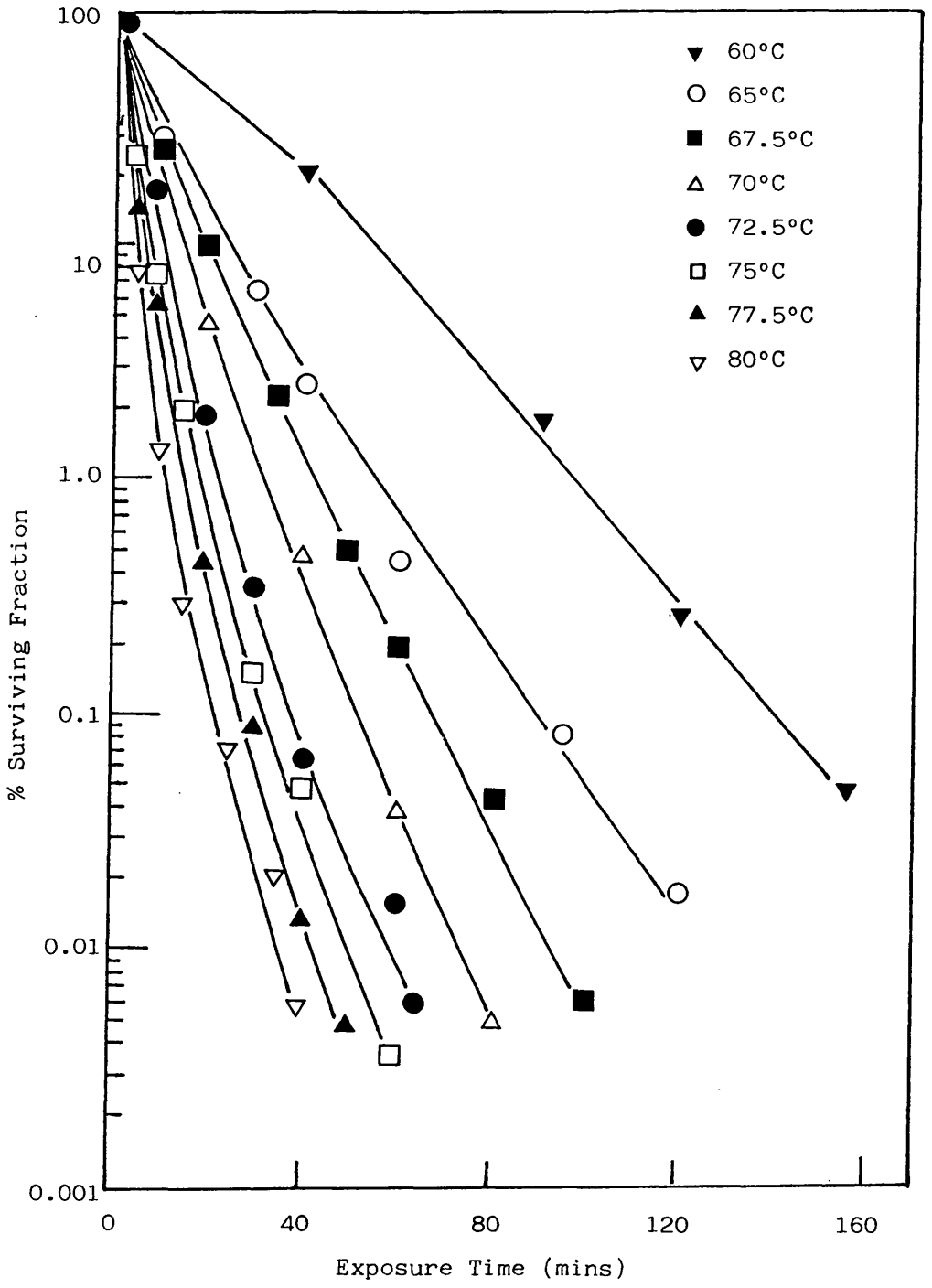


Figure 22. Effect of temperature on the inactivation of spores of B. stearothermophilus NCIB 8224 produced on SSMAVIT by 0.5% w/v formaldehyde in aqueous solution.

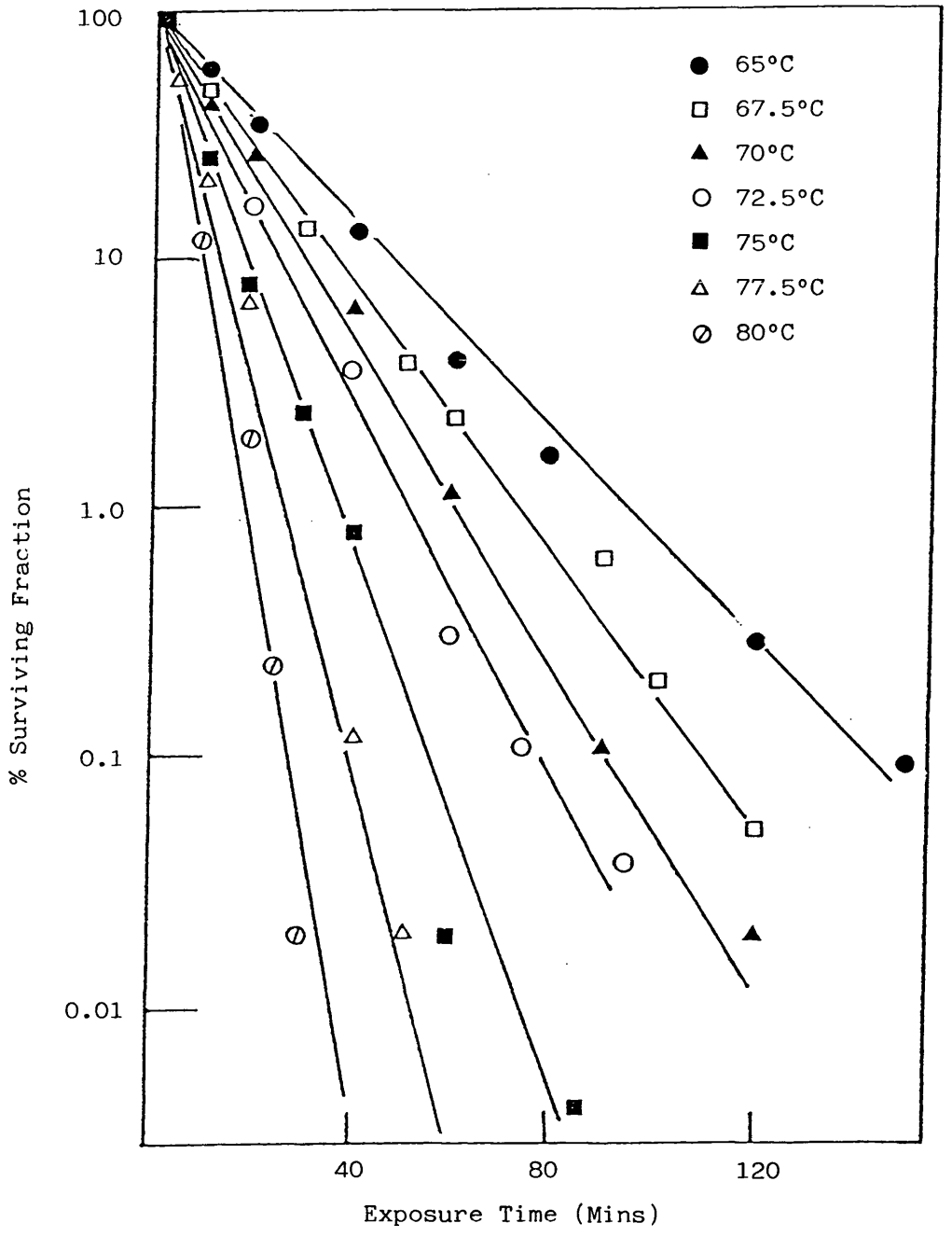


Figure 23. Effect of temperature on the inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium by 0.5% w/v formaldehyde in aqueous solution.

inactivation of bacterial spores by chemical agents often reveal evidence of tailing at prolonged exposure periods. It is therefore possible that survivor curves obtained in this investigation that deviated from the Type C shape, could be an artefact of a condensed linear scale. Expanding the linear scale could show the survivor curves to be entirely Type C.

As would be expected, increase in the exposure temperature resulted in an increase in the rate of inactivation of all the spores tested. However, the observed increase in the rate of spore inactivation with increasing exposure temperature is not consistent over equal incremental temperature increases. Obvious breaks in the survivor curves are apparent at survivor curves corresponding to temperatures between 70° and 75°C. The break is shown most clearly with spores of B. stearothermophilus NCTC 10003 produced on solid SSMAVIT medium (Fig. 19) where it occurs between 67.5° and 70°C and with spores of B. stearothermophilus NCIB 10814 produced on solid SSMAVIT medium (Fig. 20) where it occurs between 72.5° and 75°C.

In inactivation investigations where temperature is a variable the results are often described by the Arrhenius relationship (equation 6, Section 1.4.3) and it can be seen from the logarithmic form (equation 7, Section 1.4.3) that if log k is plotted against the reciprocal of the absolute temperature (1/T), a straight line Arrhenius plot of slope -Ea/2.303R is obtained from which the activation energy (Ea) can be calculated (11). Assuming log-linear inactivation kinetics, the reciprocal of the D-value is

equivalent to the inactivation rate constant 'k'. The reciprocal of the t_3-value (Section 4.4.1.1) can therefore be considered as representative of the average inactivation rate constant over three log cycles for survivor curves deviating from the log-linear pattern. Figure 24 illustrates the derivation of the t_3-value from a survivor curve for the inactivation of spores of B. stearothermophilus NCTC 10003 produced on solid SSMAVIT medium when exposed to 67.5°C suspended in 0.5% w/v aqueous formaldehyde solution.

To illustrate more clearly the phenomenon that was observed in the survivor curves following the inactivation of spores of the selected Bacillus strains by 0.5% w/v aqueous formaldehyde solution at temperatures between 60°C or 65°C and 80°C, a derived plot was constructed. The reciprocal of the t_3-value was plotted on a log scale, against the reciprocal of the corresponding absolute exposure temperature on a linear scale. The resulting plot was termed the "pseudo-Arrhenius" plot. As with the true Arrhenius plot, this derived plot would be expected to be linear with a negative slope provided that the mechanism of inactivation of the spores remains the same over the temperature range examined.

Data displayed in Figures 25-30 show the "pseuo-Arrhenius" plots derived from the data displayed in Figures 17-23 for the inactivation of spores of the three B. stearothermophilus strains produced on solid SSMAVIT and C-Ltd media. The breaks that were observed in the pattern of the survivor curves show clearly on the

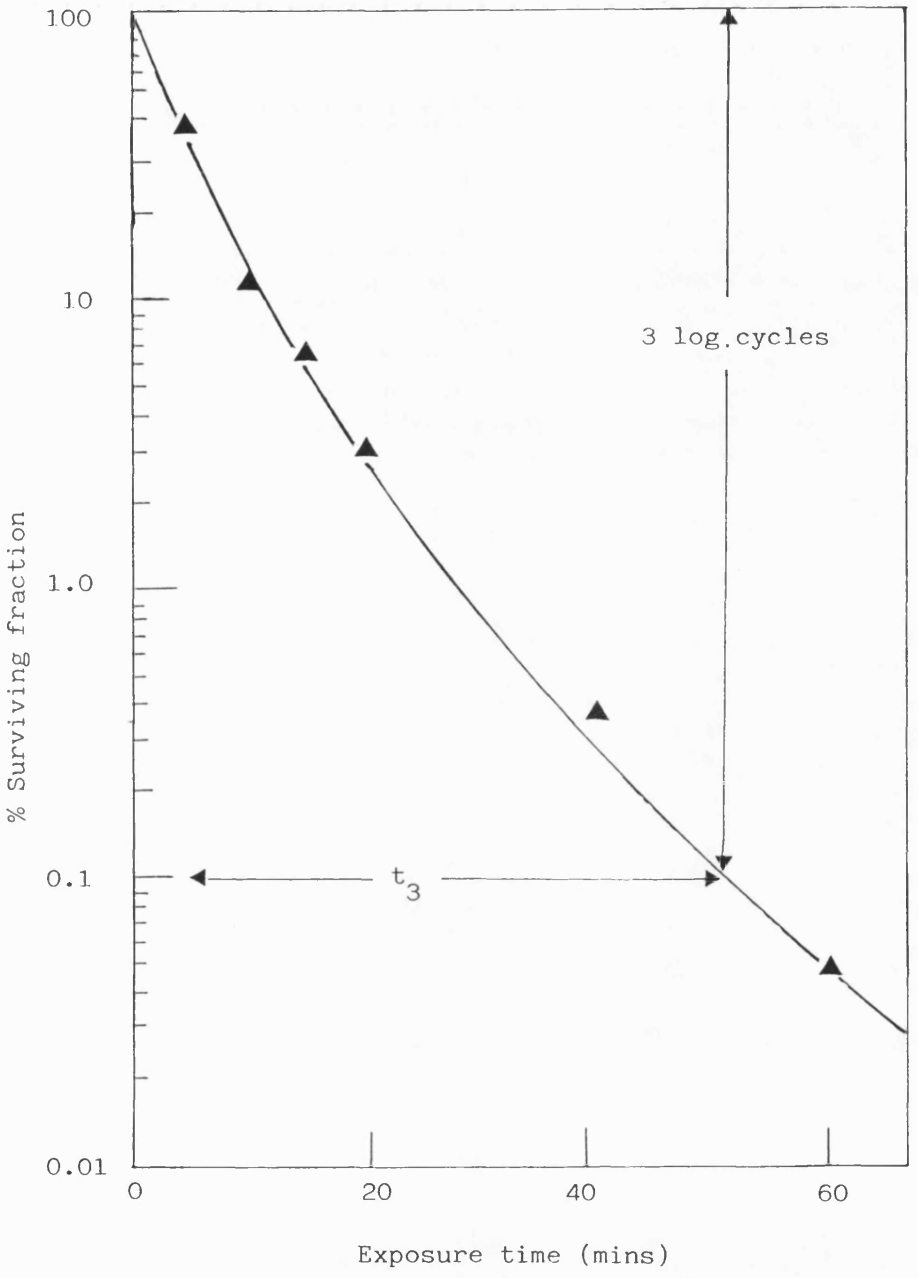


Figure 24. Survivor curve for the inactivation of spores of B. stearothermophilus NCTC 10003 produced on C-Ltd medium by 0.5% w/v formaldehyde in aqueous solution at 67.5°C illustrating the derivation of the t_3-value.

"pseudo-Arrhenius" plots as inflexions or discontinuities. For the purposes of comparison, the "pseudo-Arrhenius" plot for inactivation by 0.5% w/v aqueous formaldehyde solution of spores of the mesophilic strain B. subtilis Trav 5230, produced on solid SSMAVIT medium, is shown in Fig. 31. This is reproduced from the inactivation studies reported by Hoxey (22).

When spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium were exposed to 0.5% w/v aqueous formaldehyde solution at temperatures between 65°C and 80°C, log-linear survivor curves were obtained over the entire exposure temperature range tested (Fig. 23). In this case, the reciprocal of the t_3-value, multiplied by 3, is equal to the inactivation rate constant 'k' and a true Arrhenius plot can be constructed. This is shown in Fig. 32. Activation energies and frequency factors can therefore be calculated from the two linear sections of the plot and are recorded in Table 17. The significance of these data and of the discontinuities in the "pseudo-Arrhenius" plots is discussed in Section 5.4.

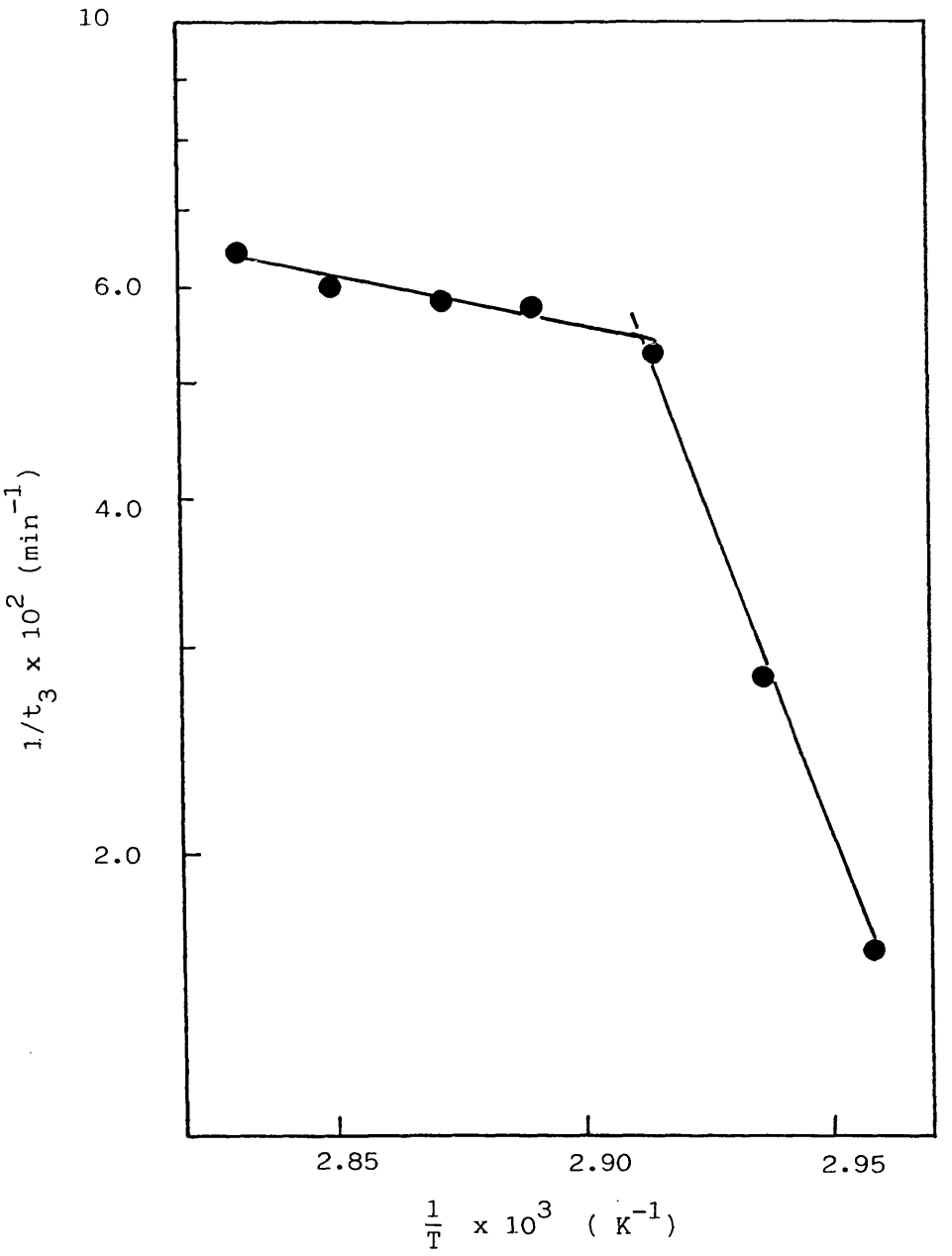


Figure 25. Pseudo-Arrhenius plot of the reciprocal of t_3 value, on a log scale, against the reciprocal of the Absolute temperature for the inactivation of spores of B. stearothermophilus NCTC 10003 produced on C-Ltd medium by 0.5% w/v formaldehyde in aqueous solution.

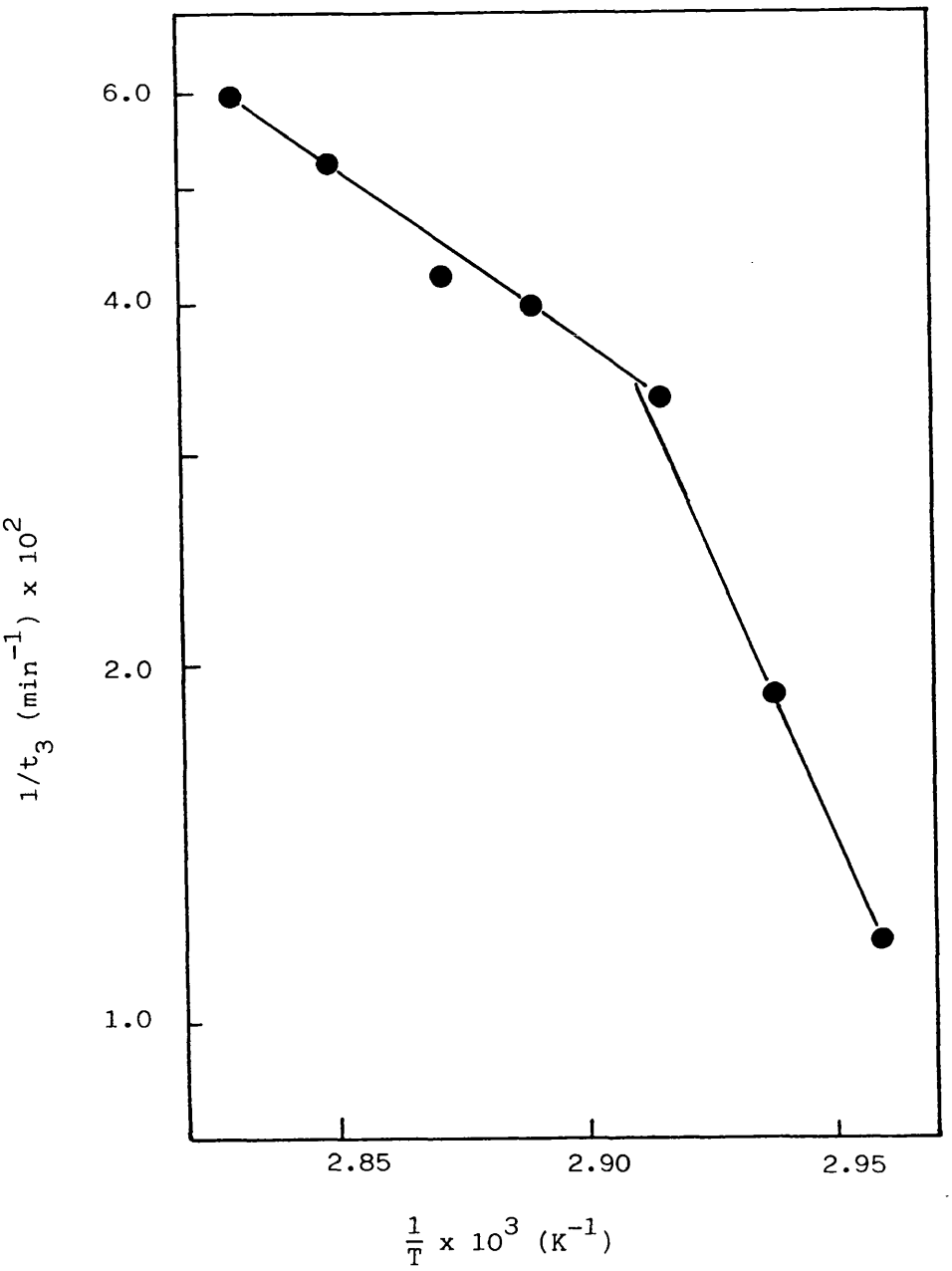


Figure 26. Pseudo-Arrhenius plot of the reciprocal of the t_3-value on a log scale, against the reciprocal of the Absolute temperature for the inactivation of spores of B. stearothermophilus NCTC 10003 produced on SSMAVIT medium by 0.5% w/v formaldehyde in aqueous solution.

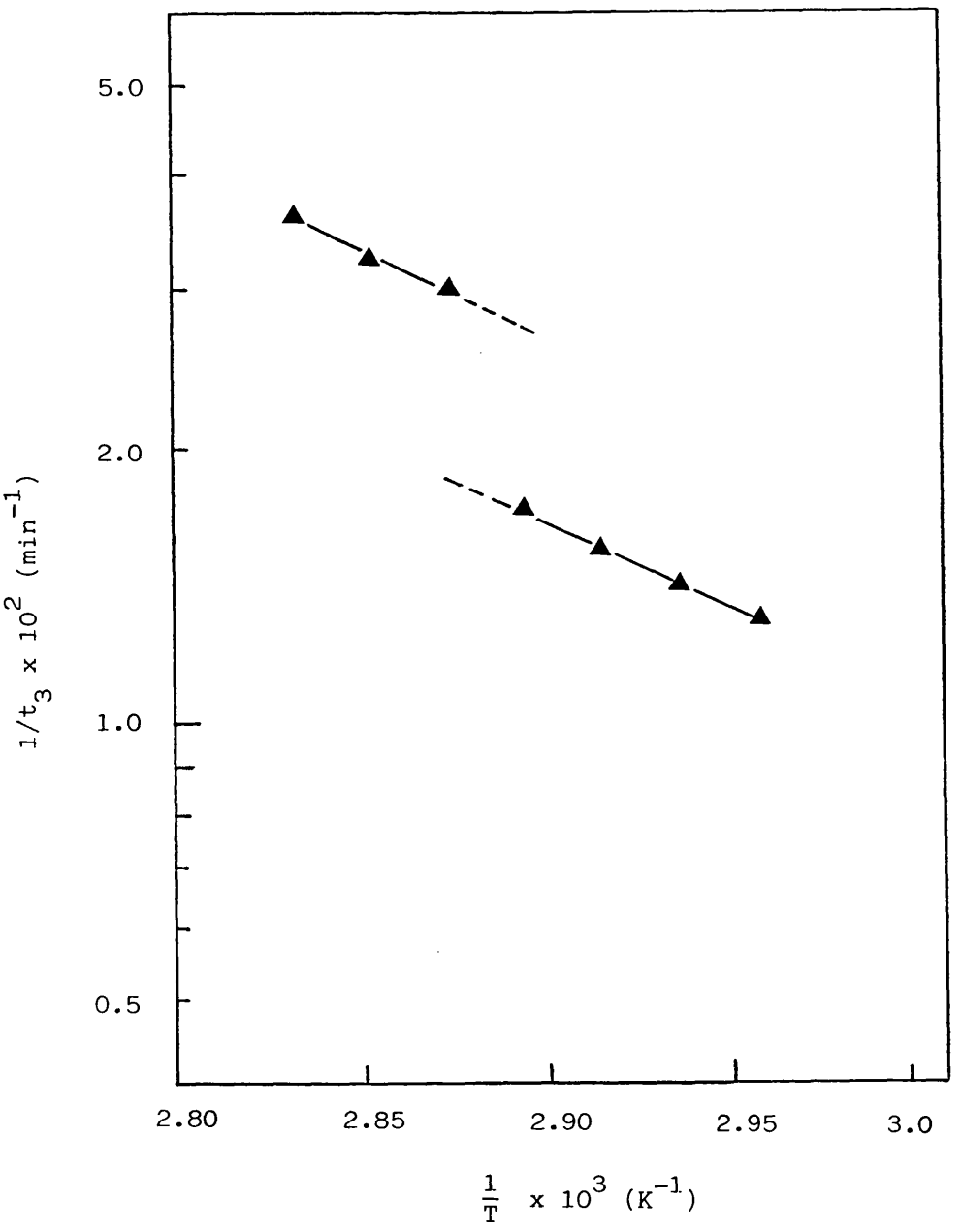


Figure 27. Pseudo-Arrhenius plot of the reciprocal of the t_3 value, on a log scale, against the reciprocal of the Absolute temperature for the inactivation of spores of B. stearothermophilus NCIB 10814 produced on SSMAVIT medium by 0.5% w/v formaldehyde in aqueous solution.

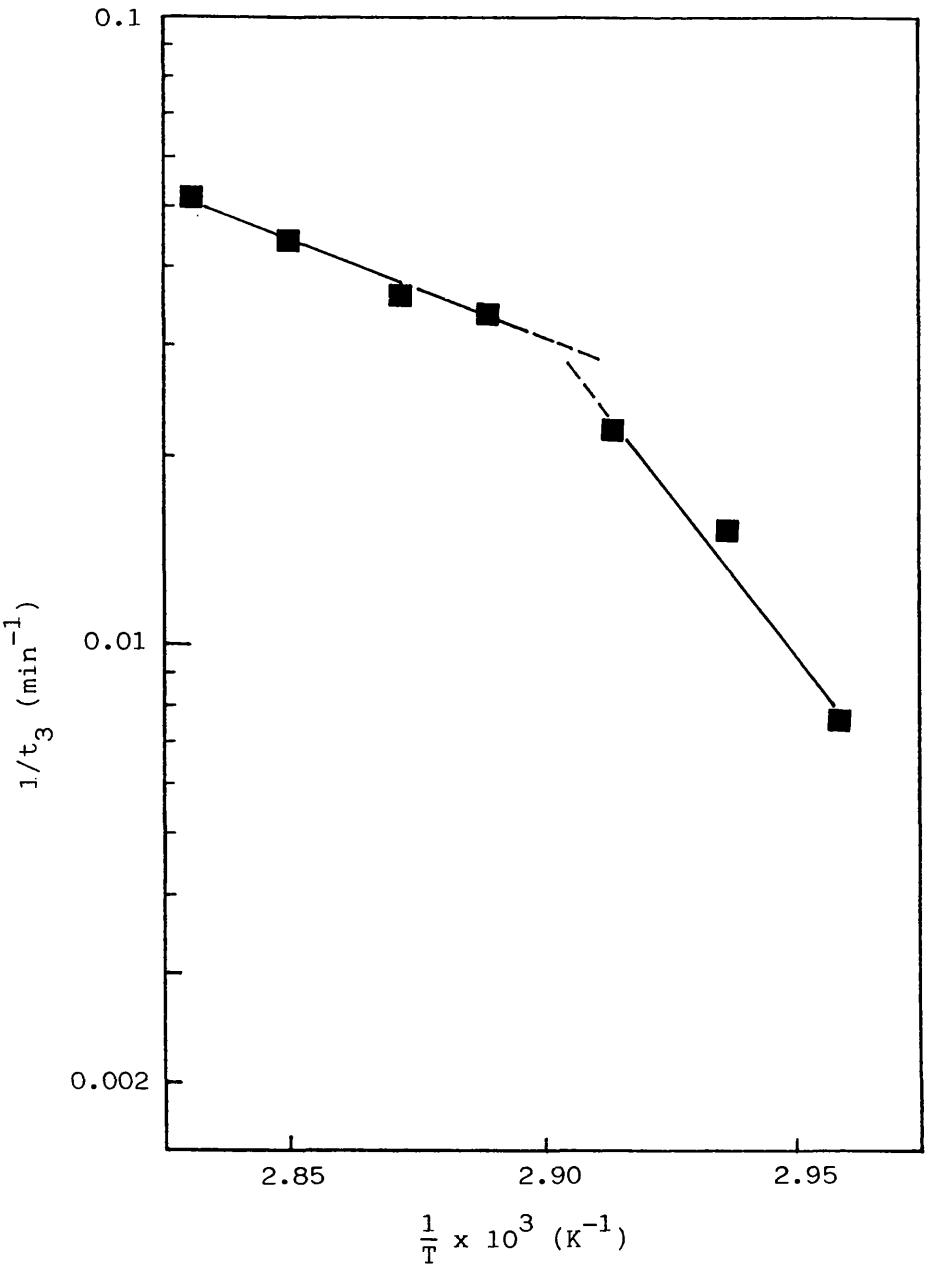


Figure 28. Pseudo-Arrhenius plot of the reciprocal of the t_3-value on a log scale, against the reciprocal of the Absolute temperature for the inactivation of spores of B. stearothermophilus NCIB 10814 produced on C-Ltd medium by 0.5% w/v formaldehyde in aqueous solution.

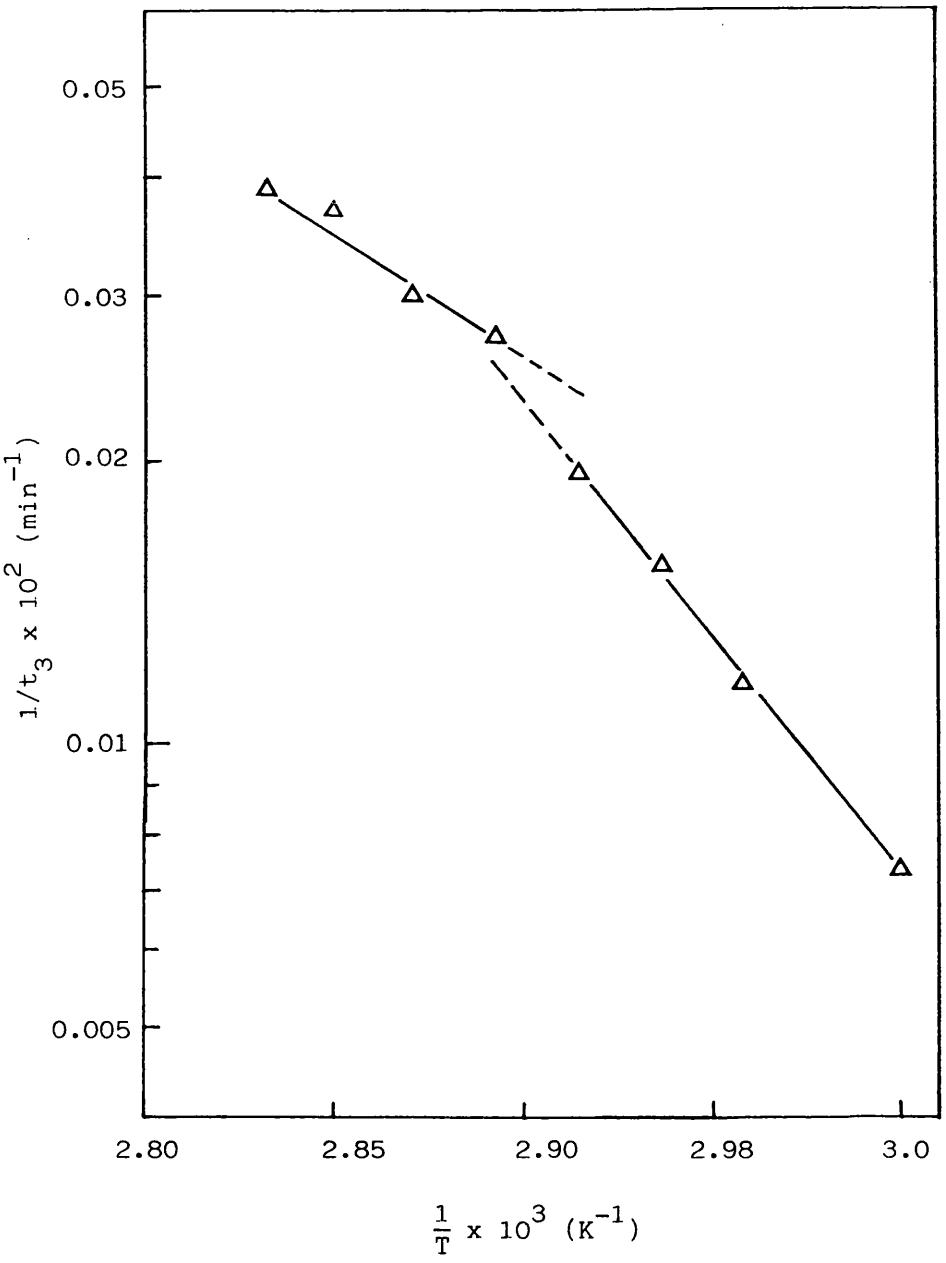


Figure 29. Pseudo-Arrhenius plot of the reciprocal of the t_3-value on a log scale, against the reciprocal of the Absolute temperature for the inactivation of spores of B. stearothermophilus NCIB 8224 produced on SSMAVIT medium by 0.5% w/v formaldehyde in aqueous solution.

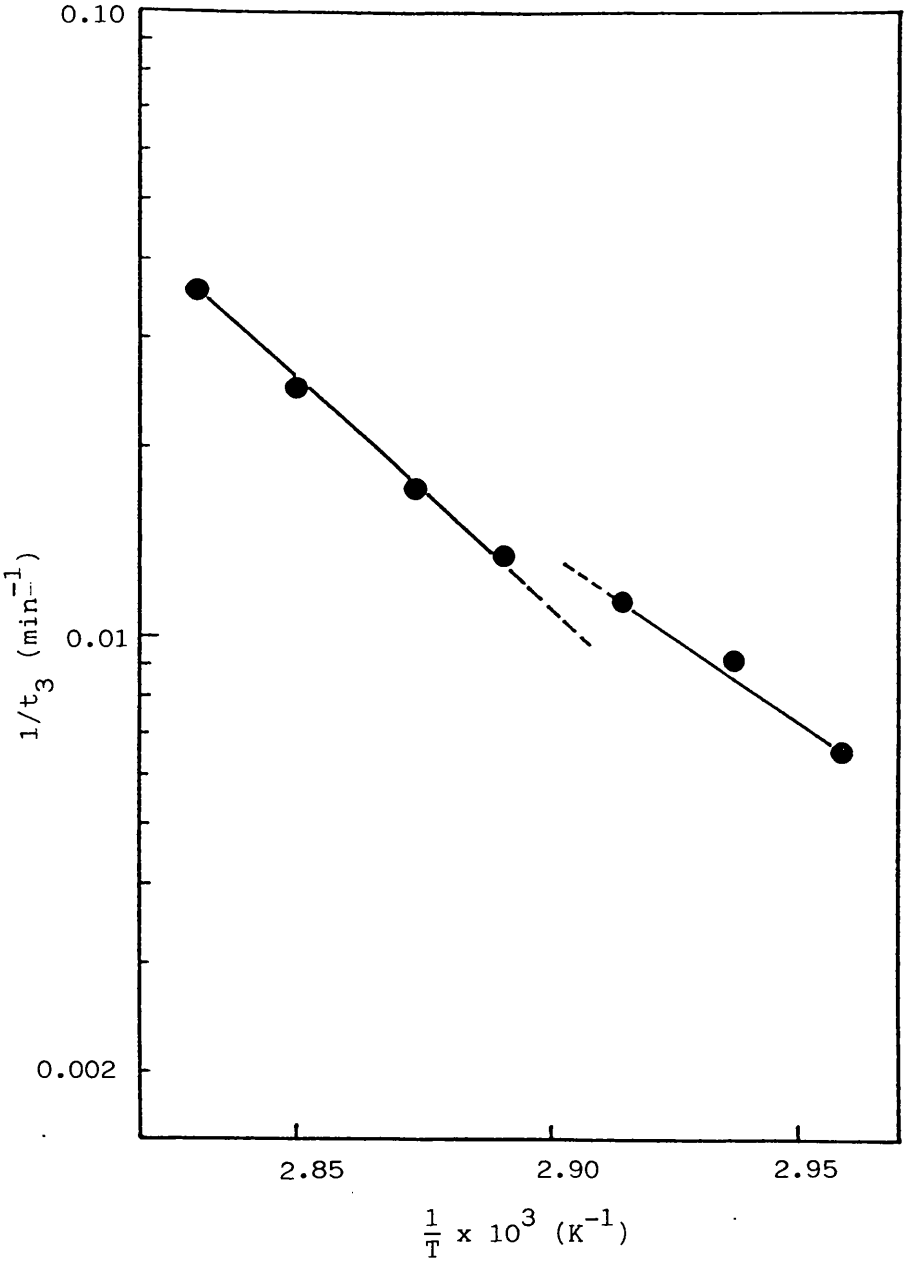


Figure 30. Pseudo-Arrhenius plot of the reciprocal of the t_3-value on a log scale, against the reciprocal of the Absolute temperature for the inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium by 0.5% w/v formaldehyde in aqueous solution.

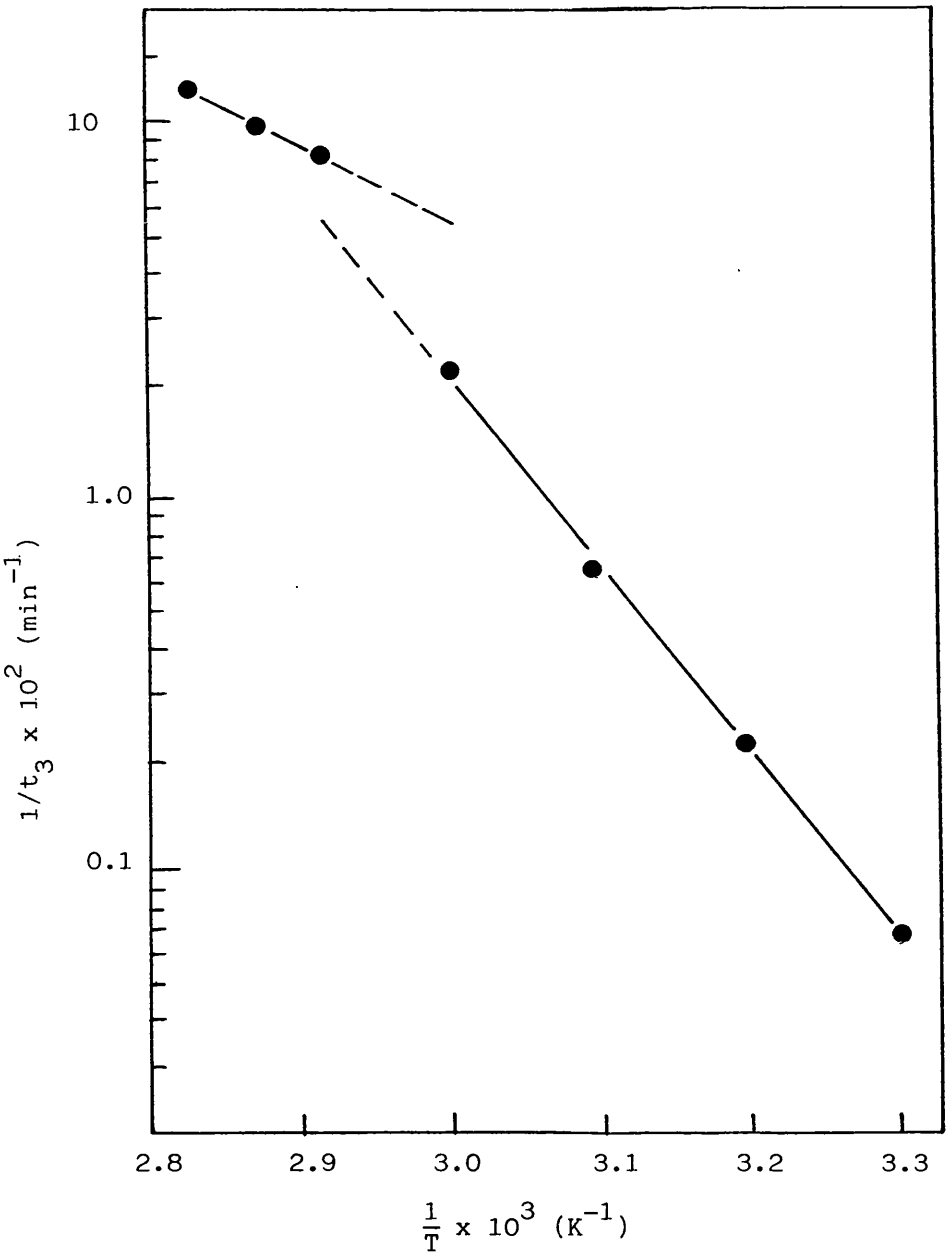


Figure 31. Pseudo-Arrhenius plot of the reciprocal of the t_3-value on a log scale, against the reciprocal of the Absolute temperature for the inactivation of spores of B. subtilis Trav 5230 produced on SSMAVIT medium by 0.5% w/v formaldehyde in aqueous solution. Reproduced from data reported by Hoxey (22).

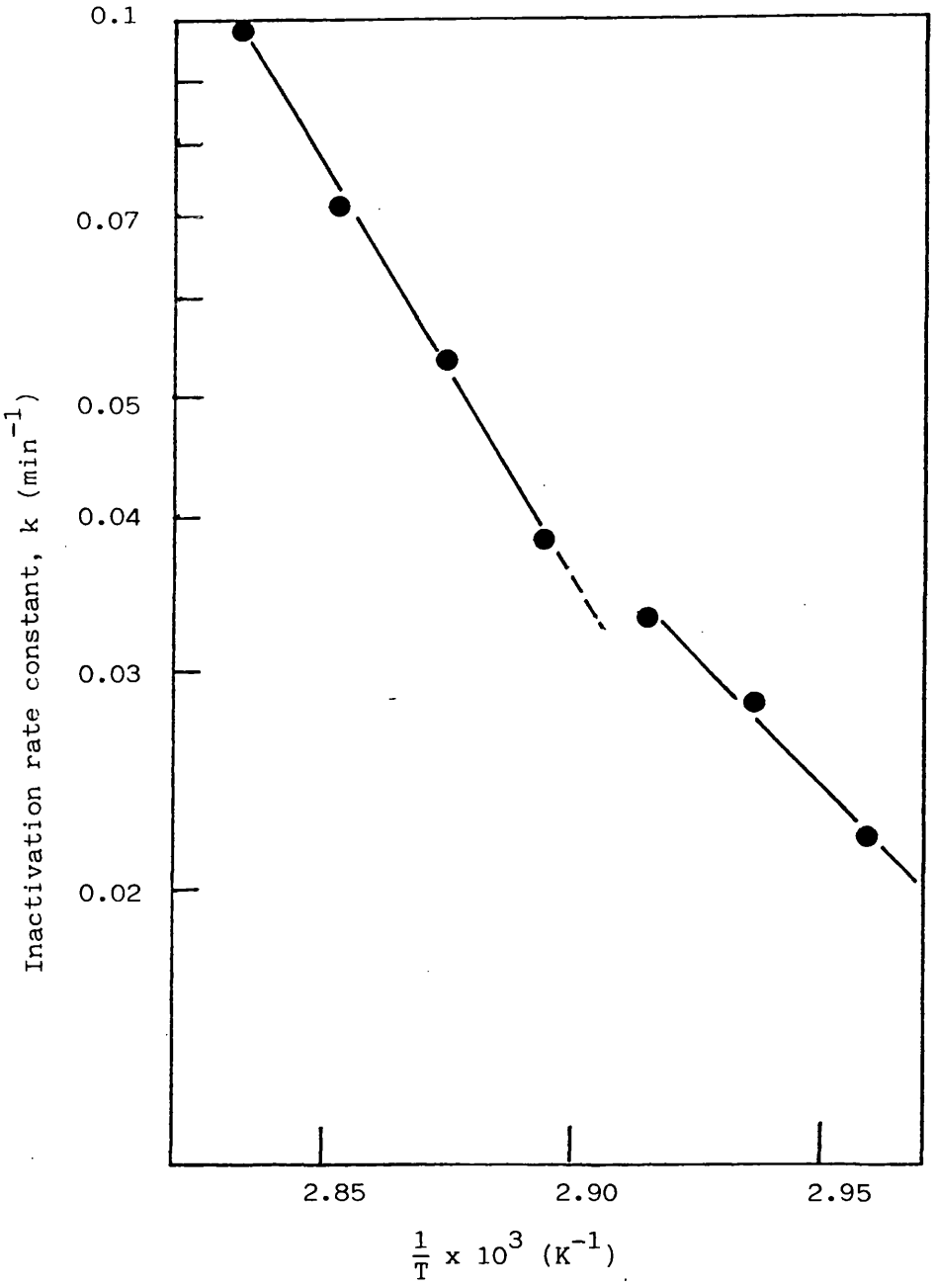


Figure 32. Arrhenius plot of inactivation rate constant, k, on a log scale against reciprocal of Absolute temperature for the inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium by 0.5% w/v formaldehyde in aqueous solution.

Table 17. Activation Energies and Frequency Factors Dervied from Arrhenius Data for the Inactivation of Spores of B. stearothermophilus NCIB 8224 produced on C-Ltd Medium by 0.5% w/v Aqueous Formaldehyde Solution at 65° to 80°C

Temperature Range (°C)	Activation Energy ($kJmol^{-1}$)	Frequency Factor (A)
65-70	78.21	1.2
72.5-80	127.9	1.3

5.3.4 Effect of Cold Room Storage on the Resistance of Bacterial Spores to 0.5% w/v Aqueous Formaldehyde Solution at 70°C

Bacterial spores to be used as biological indicators for validation and monitoring of sterilization processes have not only to be of consistent and appropriate resistance to sterilants but should in addition be stable in their characteristics upon storage. Spores may be stored for considerable periods before they are used and therefore their stability with respect to their response to inactivating treatments is yet another prerequisite which needs to be established. An ideal monitor organism must be sufficiently stable so that once monitors are prepared, they can be stored for realistic periods without alteration in their reliability to monitor the sterilization process. Information relating to the effects of storage on the resistance of spores to chemicals is very scarce (214). The published data on effects of storage on heat

resistance of bacterial spores is often contradictory (79, 215). It has been suggested by Waites and Bayliss (214) that resistance to chemicals may change on storage although Reich (82) reported no difference in ethylene oxide resistance of spores of B. subtilis. Consequently this study was undertaken with the intention of establishing the stability on storage of the spores of the three selected B. stearothermophilus strains produced on C-Ltd medium with respect to their response to inactivation by 0.5% w/v formaldehyde in aqueous solution at 70°C.

Aqueous spore suspensions (pH 6.2) containing approximately 1×10^8 viable spores/ml were stored in 1.5 ml aliquots sealed in sterile 2 ml glass ampoules at 4°C in the dark. The resistance to 0.5% w/v formaldehyde in aqueous solution at 70°C was then determined at intervals over a 30 week storage period using the method outlined in Section 5.2.4. The t_3-values were read from each survivor curve and were plotted as a function of storage time in weeks. Figure 33 illustrates the data obtained with the spores of the three selected Bacillus stearothermophilus strains produced on C-Ltd medium. At this stage solid SSMAVIT was not considered a good choice of chemically defined media and therefore spores produced on it were not included in this investigation. The data displayed in Fig. 33 show that the resistance of the tested spores to inactivation by 0.5% w/v aqueous formaldehyde at 70°C was dependent on the storage period. Spores of B. stearothermophilus NCTC 10003 displayed the least variation with the initial resistance similar to that determined at the end of the storage period

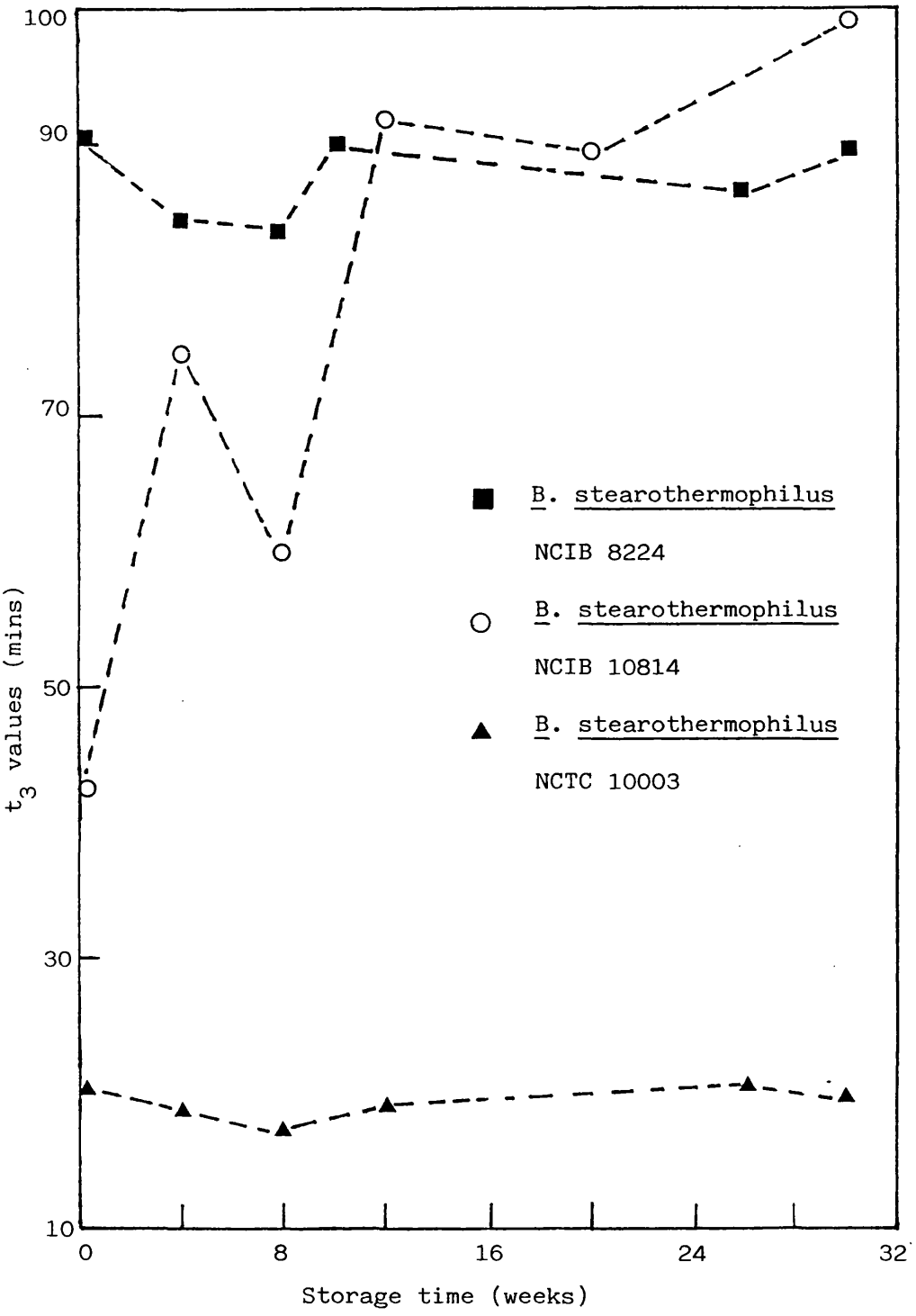


Figure 33. Variation in resistance to 0.5% w/v aqueous formaldehyde solution at 70°C during storage at 4°C of spores of B. stearothermophilus strains produced on C-Ltd medium.

(t_3 = 19.7 ± 1.14 minutes). Spores of B. stearothermophilus NCIB 8224 showed an initial loss of resistance but subsequently regained the level of resistance observed prior to storage and maintained this level during the remainder of the 30 week storage period. The variation in the t_3-value during storage (t_3 = 88 ± 3.0 minutes) was considered insignificant. Spores of B. stearothermophilus NCIB 10814 showed a marked increase in resistance to 0.5% w/v aqueous formaldehyde solution at 70°C with storage. The t_3-value at 30 weeks (99 minutes) was more than double the value obtained at the beginning of the storage period (44 minutes).

5.4 DISCUSSION

Data on the reproducibility of resistance to inactivation by 0.5% w/v aqueous formaldehyde solution at 70°C within and between batches of spores of B. stearothermophilus NCIB 10814 produced on SSMAVIT medium (Figs. 14-15), demonstrate the reproducibility of the method. This, together with the reproducibility of Germination Index (Chapter 3) and reproducibility in response to moist heat inactivation (Chapter 4) is further confirmation that spores of consistent characteristics can be produced from chemically defined sporulation media. Reproducibility, especially in response to the disinfection or sterilization conditions will ensure successful monitoring of process cycles, with the same safety margin. Assuming the behaviour of spores in aqueous formaldehyde is similar to their behaviour when exposed to gaseous formaldehyde the strain exhibiting the highest reproducible resistance to inactivation by

aqueous formaldehyde solution will be desirable provided it satisfies the other requirements for an ideal biological indicator organism.

The resistance of the spores of the selected Bacillus strains to inactivation by 0.5% w/v formaldehyde in aqueous solution was determined at 70°C since most of the LTSF cycles are reported to operate at temperatures around 70°C (2). A value of 0.5% w/v was representative of the formaldehyde concentration of 25 mg l^{-1} commonly used in UK LTSF cycles (74). A range of sensitivities and a variety of survivor curve types were displayed by the spores of the strains tested (Fig. 16). The range of sensitivities and variety of survivor curve shapes shown by spores of the three Bacillus stearothermophilus strains when grown on each chemically defined medium is illustrative of the heterogeneity of the taxonomic group (216). When reporting on Bacillus stearothermophilus species it is therefore necessary to qualify the strain and production conditions since the group is made up of vast numbers of strains that cover a wide range of sensitivities to lethal agents. These observations are also in agreement with reports of Baylis et al. (199) that spores of different strains of the same species and spores of different species can exhibit different resistances to the same chemical. The composition of sporulation media has been shown in previous chapters to influence growth, sporulation, germination index and moist heat resistance of the bacterial spores tested. The results in Fig. 16 and the data displayed in Table 16 show that resistance to chemicals, in this case 0.5% w/v aqueous

formaldehyde at 70°C, is similarly influenced by the composition of the sporulation medium. Linear inactivation kinetics to these test conditions were displayed by spores of B. stearothermophilus NCIB 8224, NCIB 10814 both grown on C-Ltd media, strain NCTC 10003 from SSMAVIT medium and by spores of B. subtilis Trav 5230 grown on SSMAVIT (Fig. 16). Spores of B. stearothermophilus NCTC 10003, when produced on C-Ltd medium, exhibited a survivor curve showing an initial shoulder followed by a steep slope. The remaining strains of B. stearothermophilus showed evidence of tailing at prolonged exposure periods. In general, as would be expected, spores of the thermophilic B. stearothermophilus strains were more resistant to the test conditions than spores of the mesophilic B. subtilis strain. A biological indicator organism intended for monitoring processes involving formaldehyde will have to be selected from the thermophilic group and has to display linear inactivation kinetics. The orders of increasing spore resistance to either moist heat at 110°C or to 0.5% w/v aqueous formaldehyde solution at 70°C are quite different. The data displayed in Table 18 summarises the observed characteristics of the spores of the selected Bacillus species from which the potential biological indicator organism can be recommended.

The data shown in Table 18 demonstrate that with bacterial spores, resistance to one agent is not necessarily the same as the resistance to other treatments implying different mechanisms of inactivation. Waites (217) reported that spores with extreme heat resistance are not especially resistant to chemicals suggesting

Table 18. Data summarising the influence of sporulation media on Germination Index and the resistance of spores of *Bacillus* species to inactivation by moist heat at 110°C and 0.5% w/v aqueous formaldehyde solution at 70°C

Strain	Sporulation Medium (solid)	G.I. (%)	t_3-value (mins) moist heat (110°C)	t_3-value (mins) Aqueous formaldehyde solution (0.5% w/v, 70°C)
B. stearothermophilus NCIB 10814	SSMAVIT	17	75	84
	C-Ltd	3	120	43
B. stearothermophilus NCIB 8224	SSMAVIT	25	28	68
	C-Ltd	68	37	90
B. stearothermophilus NCTC 10003	SSMAVIT	83	14	30
	C-Ltd	78	8	20
B. subtilis Trav 5230	SSMAVIT	5.7	7	10

that dehydration of the protoplast, which is important in conferring heat resistance on spores, is less important in making spores resistant to chemicals. This is supported by the observation that spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium were more sensitive to inactivation by moist heat at 110°C (t_3 = 37 mins) than spores of strain NCIB 10814 (t_3 = 120 mins). However, when exposed to 0.5% w/v aqueous formaldehyde solution at 70°C, spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium were more resistant (t_3 = 90 mins) than spores of B. stearothermophilus NCIB 10814 produced on C-Ltd medium (t_3 = 43 minutes).

C-Ltd medium produced a high yield (95%) of B. stearothermophilus NCIB 8224 spores in minimal incubation time of 48 hrs (Chapter 3). This strain also exhibited a high Germination Index (68%). Spores of this strain when produced on C-Ltd medium were the second most resistant to inactivation by moist heat at 110°C. Spores of B. stearothermophilus NCIB 10814 produced on both CDM where the most resistant to inactivation by moist heat at 110°C but displayed low Germination indices (Table 18). Spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium were the most resistant to inactivation by 0.5% w/v formaldehyde in aqueous solution at 70°C (Fig. 16) displaying a log-linear death pattern. On the basis of this criterion, spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium can be recommended for development as biological indicators for LTSF processes. This is assuming of course that the mechanism of inactivation of bacterial

spores by aqueous formaldehyde is similar to that of gaseous formaldehyde. The exact mechanism of formaldehyde induced lethality in bacterial spores is not yet established. Consequently the basis of resistance by bacterial spores to chemicals is not understood although resistance to chlorine, octanol and ethylene oxide may be produced by the intact spore coat since spores with defective coats have remarkedly reduced resistance (217). Trujillo and David (123) claimed that sporicidal activity of aqueous solutions of formaldehyde was caused by sporostasis due to the reversible inhibition of germination. Spicher and Peters (1, 158) reported that subsequent heating reactivated spores of B. subtilis (strain unspecified) apparently killed by aqueous formaldehyde solution. This may imply that subsequent heating of the spores exposed to formaldehyde solution overcame the inhibition to germination. These experiments were carried out at 22°C and it is possible that the authors could have activated a previously dormant portion of the spore population. Investigations are underway to establish whether subsequent heating can reactivate spores of B. stearothermophilus NCIB 8224 exposed to 0.5% w/v aqueous formaldehyde solution at 70°C (159). Subsequent heating did not reactivate spores of B. subtilis var niger exposed to vapourized formaldehyde solution (218).

Steele (182) selected spores of B. stearothermophilus NCTC 10003 produced on C-Ltd medium as suitable for development as biological indicators for LTSF processes. Investigations reported in this thesis show that this strain, when produced on C-Ltd medium, produced a high spore yield (90%) in two days and exhibited

a high Germination Index (78%). However this strain was the most sensitive of the thermophillic group to inactivation by moist heat at 110°C and by 0.5% w/v formaldehyde in aqueous solution at 70°C. This strain also exhibited a Type B survivor curve when exposed to aqueous formaldehyde at 70°C. B. stearothermophilus NCTC 10003 when produced on both CDM, has further drawbacks in recovery of treated and untreated spores. The spores were observed to germinate readily but rapidly outgrew into amorphous, effuse and rhizoid colonies on both NA and TSA which can easily introduce errors in estimating survivors.

The effect of temperature on the resistance of the spores of the three B. stearothermophilus strains produced on both chemically defined media to inactivation by 0.5% w/v aqueous formaldehyde solution was reported in Section 5.3.3. There appears to be little information published about this. Trujillo et al. (123) reported the bactericidal activity of formaldehyde solutions to be markedly influenced by temperature with extensive inactivation at temperatures above 40°C. Toledo et al. (219) found that the relative resistance of different strains of B. subtilis to hydrogen peroxide varied with temperature.

In general chemicals do not inactivate bacterial spores rapidly or efficiently at ambient temperatures. However this inactivation potential may be improved by combinations with other chemicals or with physical agents (220) Such combinations offer the possibility of reducing the severity of treatment and allowing the

sterilization of thermolabile materials.

Results obtained in this investigation following the exposure of the selected spores to 0.5% w/v aqueous formaldehyde solution at various temperatures between 60° and 80°C showed that the spores progressively became more sensitive as the temperature of inactivation was increased (Figures 17-23). These determinations were carried out in unbuffered formaldehyde solutions (pH = 4.5 at room temperature). The likely change in pH caused by the different exposure temperatures was considered less likely to affect the biocidal properties of aqueous formaldehyde solution since pH fluctuations between 3.6 and 7.8 have no effect on the proportion of the monomeric and polymeric hydrates (221). In this investigation, the increased temperature, which in turn increased the synergistic inactivation potential of formaldehyde and heat, did not produce consistent decreases of spore resistance with equal incremental rises. The use of the derived "pseudo-Arrhenius" plot revealed regions of inflexions or discontinuities at temperatures where the breaks in the pattern of the survivor curves were observed (Figures 25-30).

Spores of B. stearothermophilus NCTC 10003, produced on both C-Ltd and SSMAVIT media revealed inflexions in regions corresponding to 70°C to 72.5°C (Figures 25-26). The same pattern was observed for spores of B. stearothermophilus NCIB 10814, which when grown on SSMAVIT media revealed a discontinuity in the plot at regions corresponding to temperatures of 72.5°C to 75°C (Fig. 27).

An inflexion at regions corresponding to temperatures between 70°C and 72.5°C was observed for spores of B. stearothermophilus NCIB 10814 produced on C-Ltd medium (Fig. 28). Spores of B. stearothermophilus NCIB 8224 produced on SSMAVIT medium showed an inflexion at regions corresponding to temperatures between 72.5°C and 75°C (Fig. 29) and when produced on C-Ltd medium, a discontinuity was apparent at regions corresponding to temperatures 70°C and 72.5°C (Fig. 30). This feature does not seem to be unique to spores of B. stearothermophilus strains. Hoxey (22) observed with spores of B. subtilis Trav 5230 that a discontinuity was apparent at regions corresponding to 70°C and 75°C (Fig. 31). These irregularities in the "pseudo-Arrhenius" plots were apparent for all strains tested irrespective of the sporulation media, at temperatures within 70°C and 75°C. Composition of sporulation media could have influenced the results as indicated by the different shapes of the "pseudo-Arrhenius" plots obtained for each strain when produced from different CDM. These observations were encountered by Friesen and Anderson (66) who reported on an inflexion at regions corresponding to 100° and 105°C on the Arrhenius plot following moist heat inactivation of spores of B. stearothermophilus NCTC 10003 produced on CDM.

In a chemical reaction, such breaks in the Arrhenius parameters could be explained in terms of a change in the reaction mechanism. Assuming first order death kinetics of the bacterial spores to the test conditions, these breaks could be indicative of changes in the mechanism of formaldehyde induced lethality in

bacterial spores. It is also possible that elevated temperatures could be causing structural changes of the spores, probably altering permeability thereby interfering with the availability of formaldehyde to the target molecule.

Activation energies (Ea) for the distinct phases were calculated from the Arrhenius plot (Fig. 32) for spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium exposed to 0.5% w/v aqueous formaldehyde solution at temperatures between 60°C and 70°C, the activation energy required for inactivation was 78.2 kJ/mole and for elevated temperatures between 72.5°C and 80°C a higher value of 127.9 kJ/mole was necessary. Activation energies in the order of 177 kJ/mole for moist heat inactivation of spores of B. stearothermophilus NCIB 8224 produced on SSMAVIT medium at temperatures between 70°C and 90°C were reported by Hoxey (22). These observations may suggest that the synergistic effect of 0.5% w/v aqueous formaldehyde solution and heat is more efficient at killing bacterial spores over this temperature range than moist heat alone which has a higher energy requirement for the inactivation process. At temperatures below 70°C, inactivation by aqueous formaldehyde could be due to a reaction at a single molecular site, possibly the nucleic acid bases. At higher temperatures, an additional breakdown of weak molecular bonds e.g. H-bonds could be causative of death alongside the chemical effect of formaldehyde on exposed reactive sites (66). It could be possible that at this temperature where the irregularities occur, conformational changes in the target molecule, the nucleic acids,

brought about by the heat could be shielding the reactive sites from the attack by the formaldehyde monomers (7). These observations may have some implications on the application of formaldehyde as a sterilant and on the use of bacterial spores as biological indicators to monitor LTSF processes as it is apparent that the extent of inactivation of spores by formaldehyde in aqueous solution at higher temperatures cannot be predicted from results obtained at lower temperatures. The converse is also true.

These observations could be significant in elucidating the mechanism of formaldehyde induced lethality in bacterial spores. This mechanism is currently not clearly understood. The results discussed in this chapter were obtained following inactivation of bacterial spores by aqueous formaldehyde solution at temperatures frequently employed in various LTSF protocols. Assuming the mechanism of inactivation of bacterial spores by aqueous formaldehyde solution is similar to that by vapour phase formaldehyde, then should such phenomena be countered, with inactivation of spores at LTSF conditions, these irregularities could have serious practical implications. Most LTSF cycles operate at temperatures within these regions of uncertainty. The irregularities could also explain the apparently random cycle failures that often occur with LTSF sterilizers that show no signs of mechanical failure (7).

The last experimental section of the chapter (5.3.4) described experiments performed to determine the stability of the

bacterial spores with respect to inactivation by aqueous formaldehyde at 70°C following storage in aqueous suspensions (pH 6.2-6.4) at 4°C in the dark.

Few reports have been published relating the effects of storage on resistance of bacterial spores to chemicals (214) and data published on the effect on heat resistance are often contradictory. Cook and Brown (79) and Reich et al. (80) found that spores of B. stearothermophilus, impregnated on paper strips lost heat resistance and viability after room temperature storage for periods up to 37 weeks in contrast to reports by Cook and Gilbert (81). In addition, Cook and Brown (79) found an accelerated loss of heat resistance and viability when the spore paper strips were stored over silica gel. This contradicted reports of no observed storage effect on heat resistance of lyophilized spores of B. subtilis but was in agreement with the loss in viability reported by Odlaug et al. (215). Alpine and Hodges (222) reported little changes in viability of spores of B. stearothermo- philus NCTC 10003 produced on complex and chemically defined media after storage at 22°, 4° and -18°C for periods up to 2 months but observed a small decrease in viability after further storage up to 3 months. The loss in viability at prolonged storage periods was attributed to the severe effects of the freeze drying process and clumping of spores rather than a progressive deterioration during storage. The authors also reported on progressive decrease in heat resistance at 110°C of spores of B. stearothermophilus NCTC 10003 produced on both CDM and complex media when stored frozen or freeze

dried dried for 3 months. During storage at 4° and 22°C the spores produced on CDM exhibited a progressive increase in heat resistance at 110°C and in contrast, no marked change was observed in spores produced from the complex media. In addition, Alpine and Hodges (222) also observed an increase in heat resistance of spores of B. subtilis stored under vacuum (0.02 mm Hg). Little information has been published in relation to storage effects or the chemical resistance of bacterial spores. Reich (82) observed no difference on the resistance of B. subtilis spores to inactivation by ethylene oxide after storage on paper strips at temperatures of 20° to 22°C and at -20°C for periods up to 24 months. Short-term storage (120 hrs) at controlled humidity (65 ± 15% RH) at 30 ± 2°C of B. subtilis var globigii was shown by Myers et al. (223) to have no effect on resistance to ethylene oxide and on viability. Leaper and Bloor (83) reported that spores of B. subtilis var globigii, when stored in aqueous suspensions at 4°C, showed an increased resistance to peracetic acid after long-term storage (140 weeks) but B. subtilis SA22 spores were unaffected in their resistance to hydrogen peroxide after 140 weeks storage at 4°C. The results obtained from the investigation of the effects of cold room storage of aqueous spore suspensions on spore resistance to 0.5% w/v aqueous formaldehyde at 70°C reported in this chapter show that the length of time spores are stored after production affects their resistance to inactivation by aqueous formaldehyde solution. However, this effect was more pronounced on some strains than on others (Fig. 33). Spores of B. stearothermophilus NCIB 10814, produced from C-Ltd medium, were significantly altered in their

resistance to 0.5% w/v aqueous formaldehyde at 70°C. This strain showed a gradual appreciation of its resistance over the storage period of 30 weeks at 4°C. Spores of B. stearothermophilus NCIB 8224 and NCTC 10003, both produced from C-Ltd media, did not show significant changes in resistance to the test conditions. The initial resistance values for these strains were similar to the final values after 30 weeks storage. For these two strains, storage at 4°C in aqueous suspension can be considered as having no significant effect on the resistance to 0.5% w/v aqueous formaldehyde solution at 70°C.

The mechanism by which spores express resistance to chemicals is unclear. However, the data of Gorman et al. (174) indicate that excessive washing of spore preparations leads to a reduction in heat resistance. This could be the result of interference with spore coats as suggested by the data of Waites and Baylis (54, 199) which suggest that the spore coat provides protection of B. cereus spores against action of lysozyme or chlorine. It is also possible that absorption of leachable ions from container walls could alter spore resistance upon storage. From the published literature and results expressed in this investigation, it seems inappropriate to have a general conclusion on storage effects on spore resistance to chemicals. The effect of storage on the resistance of bacterial spores to chemicals should be considered as dependent on the species, strain, sporulation conditions, storage period and the chemical in question. Stability as a characteristic of spores to be used as biological indicators

can be linked to bioindicator performance. Given the desirable characteristics already displayed by spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium, the stability in their resistance to inactivation by 0.5% w/v formaldehyde solution at 70°C during storage, makes it a strong contender for monitoring LTSF processes.

CHAPTER 6

EXPOSURE OF BACTERIAL SPORES TO LOW TEMPERATURE STEAM AND FORMALDEHYDE

CHAPTER 6. EXPOSURE OF BACTERIAL SPORES TO LOW TEMPERATURE STEAM AND FORMALDEHYDE

6.1 INTRODUCTION

The data reported in the previous chapter showed irregularities in the effects of temperature on the inactivation of spores of Bacillus species by 0.5% w/v formaldehyde in aqueous solution. The accepted temperature for LTSF sterilization in the U.K., 73°C ± 2°C, lies within the temperature range for the observed irregularities (70°C - 75°C). If the selected spores are to be of any value as biological indicators for LTSF processes, it is essential to determine whether the irregularities in response are also observed when the spores are exposed to the conditions of LTSF over the same temperature range.

To facilitate the exposure of spores to these conditions a suitable carrier system had to be developed. Materials often used as carriers on which bacterial spores are deposited include glass, porcelain, aluminium foil (224) cellulose acetate membrane filters (75) and filter papers (225). Spore resistance can greatly be influenced by the nature of the carrier materials. Impermeable surfaces tend to increase bacterial spore resistance to ethylene oxide (87) and to formaldehyde in solution (201). The degree of dehydration of the spore determines spore resistance to heat (40, 42) and ethylene oxide (87). Therefore the extent to which the spores are dried during the preparation of test pieces can affect

resistance to LTSF treatments. In order to standardize the dehydration state of the spores, it was necessary to develop a protocol for the preparation of the test pieces.

When monitoring a sterilization process, it is customary to transfer test pieces to suitable recovery broth after the sterilization process. Subsequent incubation at appropriate temperatures would result in either positive or negative growth to infer process failure or success respectively. This qualitative method is inadequate for quantitative investigation of surviving organisms since it cannot distinguish between a positive result arising from a single spore or that arising from many spores. Furthermore, false positives can be observed arising from contamination during sampling and subculturing procedures. The problem of contamination during recovery can be overcome by the use of test pieces in a sterile container containing a crushable vessel of nutrient medium (226). Shaking the container after exposure to sterilization conditions would release the nutrient which would then be inoculated with any surviving organism. Subsequent incubation at appropriate temperatures would then reveal positive or negative growth.

For experimental purposes, a quantitative recovery of surviving organisms is necessary if the results are to be expressed as survivor curves. The accuracy of estimating surviving organisms depends on the efficiency of the techniques used to physically remove spores from carrier surfaces and the subsequent ability to

count the released viable spores. Some of the methods that have been used to detach spores from carrier surfaces include homogenisation (227) or shaking with Ballotini beads (225, 228). Ultrasonication techniques have also been used to recover spores from aluminium foil strips and polymer surfaces (182, 228).

The studies reported in this thesis form part of a multidisciplinary research project into low temperature steam (LTS) disinfection and low temperature steam and formaldehyde (LTSF) sterilization. This is a continuing project which has involved a number of research workers at Bath, together with collaborations in the PHLS at Norwich and at Luton College. In order to minimise laboratory to laboratory variation in the data, it was necessary to design a standard experimental LTS/LTSF apparatus that could be used in each of these research centres. Preliminary experiments on the response of bacterial spores to gaseous formaldehyde were carried out by Hoxey (22) using a test apparatus based on a glass manifold design. For the studies reported here, it was decided to use a modified small industrial sterilizer in order to determine the response of the spores under conditions likely to exist in commercial LTSF sterilizers.

The apparatus adopted for development into the experimental test apparatus was the Miniclave 80 LTS/LTSF sterilizers (Chas F Thackray Ltd., Leeds). The Miniclave 80 design operates on the principle of admission of dry saturated steam and vapourised formaldehyde to an evacuated and humid chamber. During the 'hold

period', dry saturated steam, formaldehyde vapour and pressure are maintained at sterilizing levels. At the end of the 'hold period', there is a period of elution when residual formaldehyde vapour is removed from the chamber. The cycle is terminated by a final vacuum drying of the chamber and its contents and admission of sterile air to establish atmospheric conditions.

The Miniclave 80 was designed to be operated at set conditions by semiskilled personnel. Its successful use as an experimental test apparatus depended on the modification of the machine to enable the parameters of the LTSF process to be varied in a defined and reproducible manner. It was also necessary to design and fit into the machine a system for insertion and retrieval of spores on carriers.

The concepts, design considerations and development of the modified Miniclave 80 involved an input from each of the research workers participating in the project. A detailed description of this work has been presented by Line (229) who was responsible for the construction and testing of the final apparatus. An extract of this is given below (Section 6.2.1). Work described in this chapter also outlines the protocol developed for the preparation of test pieces and recovery of viable spores from the carriers. Section 6.3.1 describes the technique used to inactivate formaldehyde for the LTSF studies. Using a standard protocol, spores of Bacillus stearothermophilus NCIB 8224 produced on C-Ltd medium, were used to evaluate the modified apparatus when exposed to LTSF at 80°C and

16.38 mg l^{-1} formaldehyde concentration.

6.2 MATERIALS AND METHODS

6.2.1 Modifications to the Miniclave 80 LTSF Sterilizer

6.2.1.1 Steam Supply and Temperature Control

The apparatus had to be modified to operate at a range of temperatures between 60°C and 80°C for periods in excess of 1 hour. An adequate steam supply was therefore necessary and a reliable temperature control was required to avoid the creation of superheat conditions or condensation of the steam in the chamber of the Miniclave 80. The original steam vapouriser system was disconnected to be replaced by an external steam supply system. A 10 litre electrically heated portable autoclave (Gallenkamp, Loughborough) fitted with a pressurestat was used to generate a steady steam supply. The pressurestat was set at 10 lbs p.s.i. to produce dry saturated steam at 115°C. Five litres of distilled water in the steam generator was sufficient to guarantee a steady steam supply for up to ten 120-minute LTSF cycles. Steam would leave the autoclave through a permanently open ¼" ball valve inserted into the autoclave lid, and through flexible rubber tubing to the Miniclave 80 steam supply system. As manufactured, the sterilizer was fitted with a pressure switch to control the 'hold period' pressure and thus the 'hold period' temperature. This system was unsuitable where defined variation in operating temperature was

required. Steam was therefore admitted to the chamber via a steam control solenoid valve. A CAL-9000 (RS Components Ltd.) thermostat was installed to control the steam control solenoid valve. The sensing probe of the thermostat, a copper-constantine thermocouple (Comark, BS 4937J) was positioned in the main chamber drain. The CAL-9000 could be set to control the operational temperatures between 60° and 80°C (± 0.5°C). By sensing the drain temperature, the control valve would be activated to admit steam into the chamber to maintain the set temperature. An additional copper-constantine thermocouple was sited at the top of the chamber and connected to a Servogor 120 continuous chart recorder (BBC Goerz Metrawatt) to provide a record of temperature variations in the chamber during the operating cycle.

6.2.1.2 Jacket Temperature

The temperature of the thermostatically regulated electric thermal tape around the chamber was adjusted to 2°C above the CAL-9000 set operational temperature to minimise generation of superheat conditions in the chamber and also to avoid steam condensation and polymerisation of formaldehyde vapour. The jacket temperature (which was fixed at 75°C by the manufacturer) was adjusted via a temperature control potentiometer in the temperature control circuit.

6.2.1.3 Hold Period Timer

On the Miniclave 80, the timer controlling the 'hold period' was pre-set at 60 minutes by the manufacturer. This was replaced by a 215 minute timer (Omron UB MINY Type STP-NMH, O.T.E. JAPAN) to allow variation in exposure times. Survival of bacterial spores at different set conditions of LTSF could then be determined at various time intervals.

The effect of these modifications to the Miniclave 80 can be seen in Figs. 34a-b which show the temperature and pressure profiles during the operating cycle of the sterilizer before and after modification.

6.2.1.4 Formaldehyde Input and Measuring Device

The formaldehyde injection delay timer of the Miniclave 80 was factory set at 6 seconds, to allow activation of the formaldehyde entry solenoid valve and subsequent admission of 3 ml of formaldehyde solution (38% w/v) to the vapouriser. For experimental purposes, it was desirable to vary the amounts of formaldehyde injected to generate various formaldehyde concentrations in the chamber. A formaldehyde input and measuring device was designed and installed to replace the original formaldehyde input system (Fig. 35). A 10 ml graduated glass syringe barrel incorporating a Y-piece PTFE adapter tubing at its discharge end was secured on the front panel of the apparatus. One

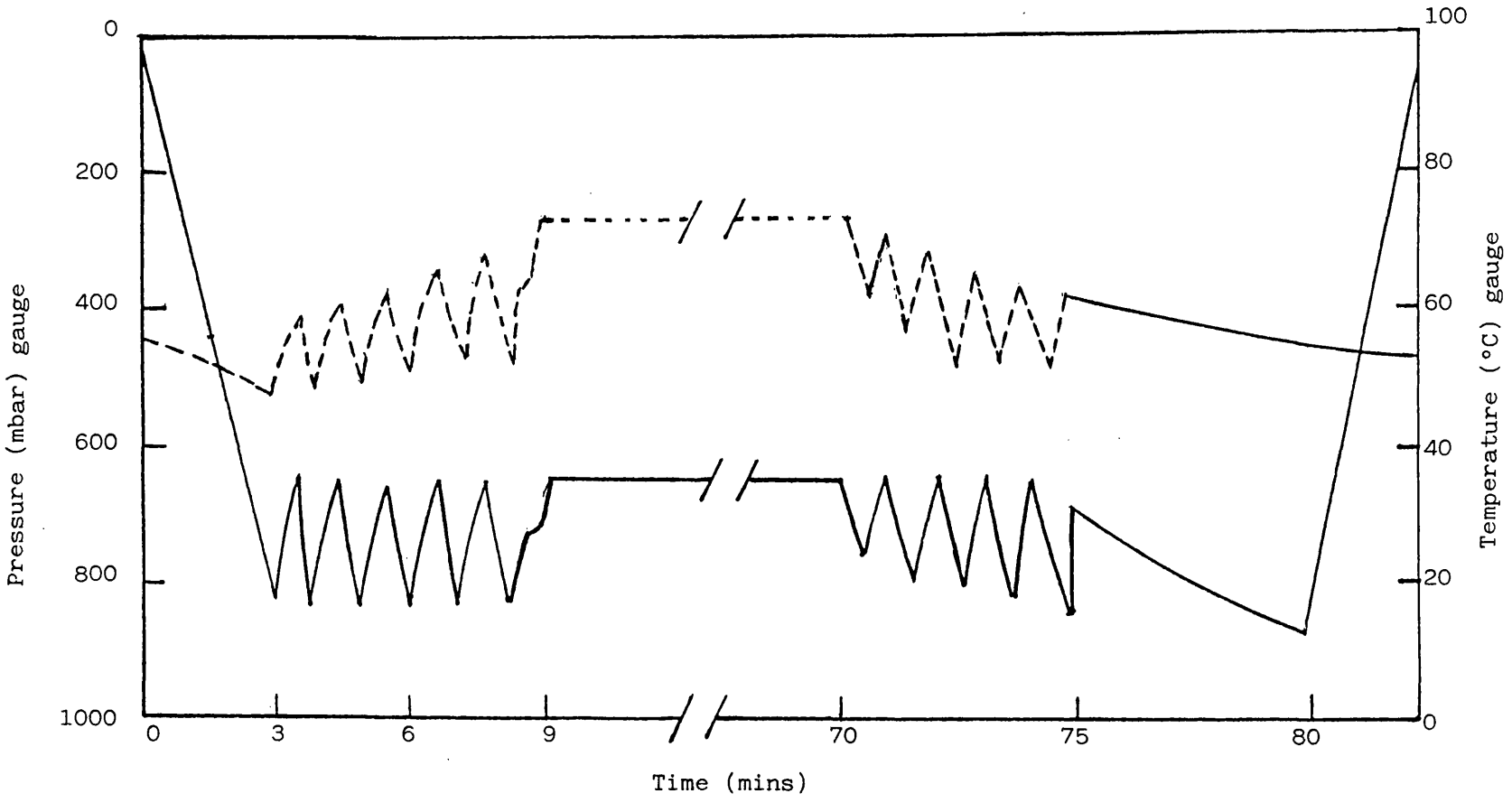


Figure 34(a). Miniclave 80 LTSF operating cycle at 73°C before modification.

Key: _____ Pressure graph _ _ _ _ _ Temperature graph

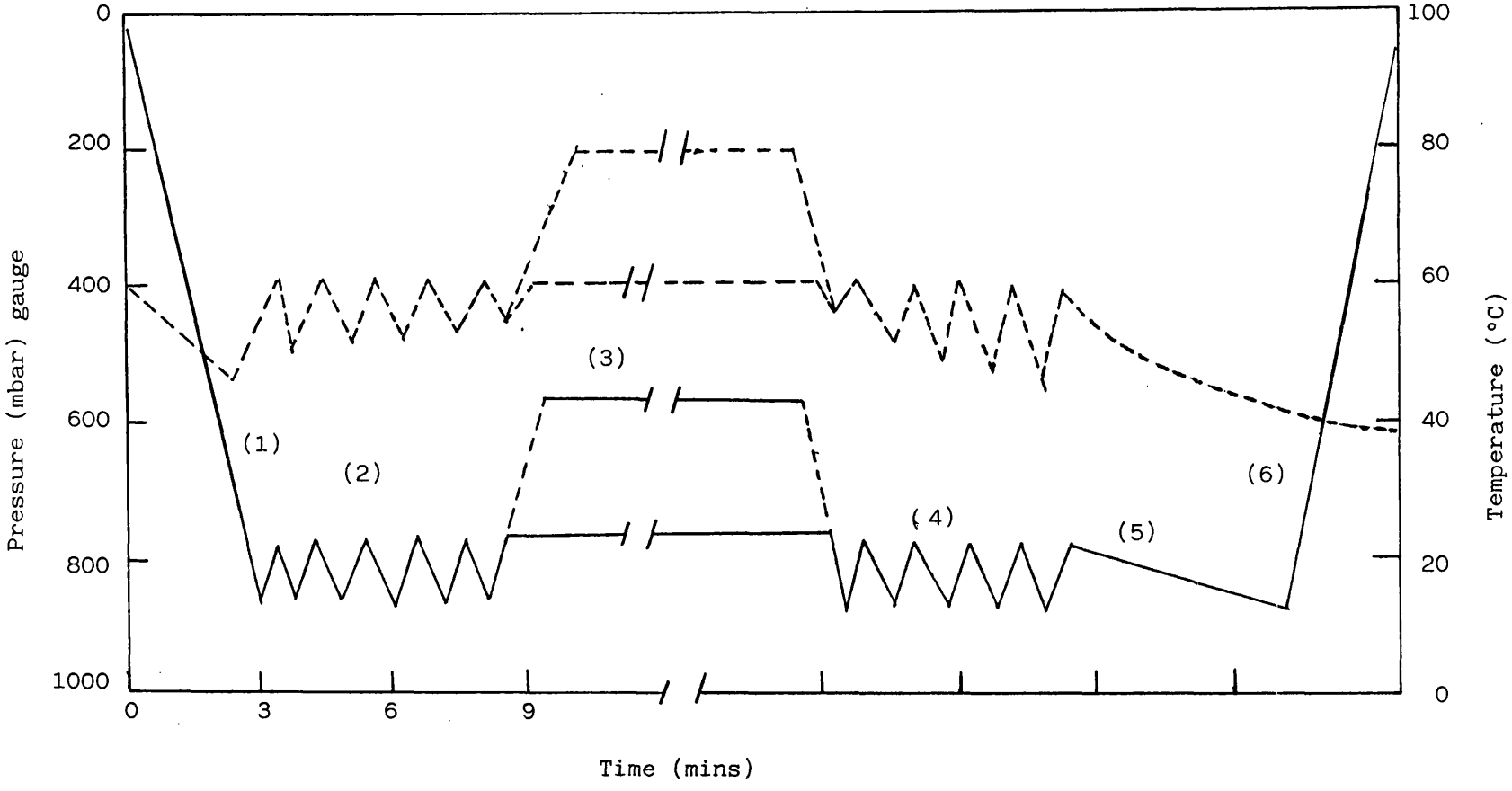


Figure 34(b). Miniclave 80 LTSF operating cycle (60° to 80°C), after modification

Key: 1. evacuation; 2.steam/vacuum pulse; 3. hold (sterilizing); 4. elution; 5. drying; 6. aeration

——— Pressure graph ------ Temperature graph

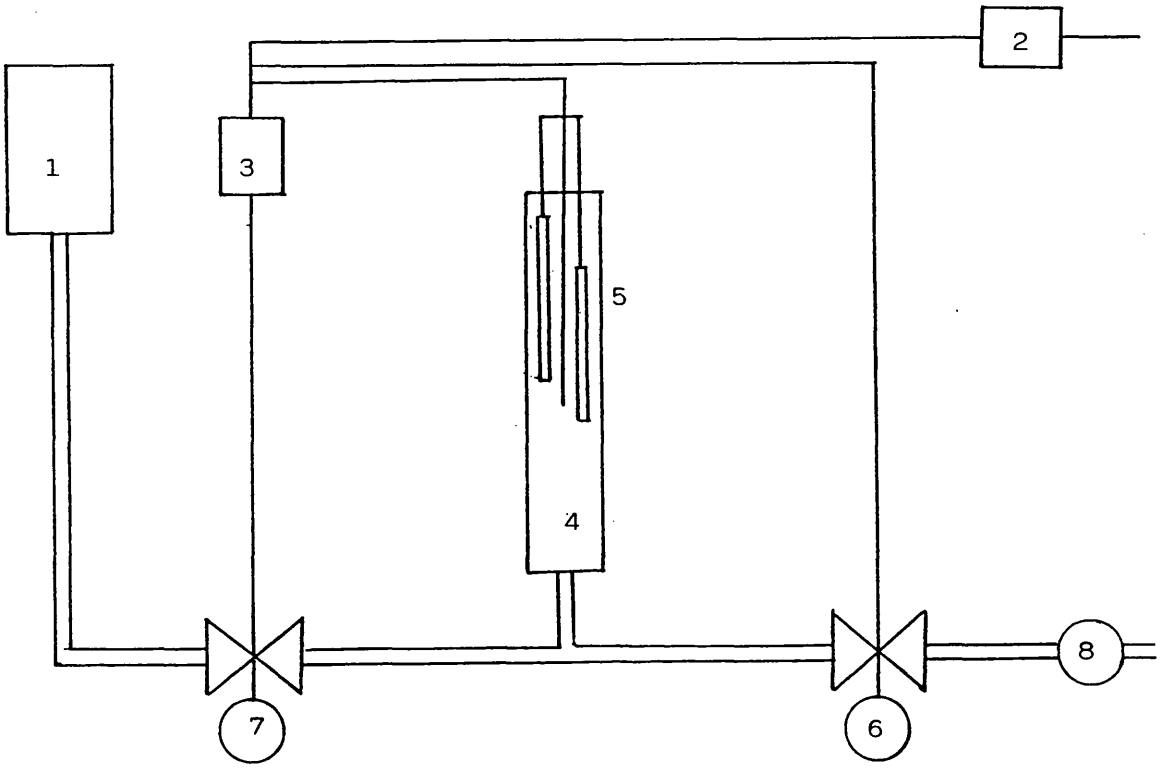


Figure 35. Pipework and circuit diagram of the formaldehyde input and measuring device showing:

1. Formaldehyde reservoir
2. Formaldehyde refilling/delay timer
3. Floatless level switch
4. Formaldehyde measuring cylinder
5. Level switch sensing probes
6. Formaldehyde entry solenoid valve
7. Formaldehyde refilling solenoid valve
8. Check valve (to vaporiser)

arm of the Y-piece was connected to the formaldehyde entry solenoid valve (6) and the other to the refilling solenoid valve (7). The formaldehyde refilling valve (7) was conected (by a 6 mm O.D. rigid PTFE tubing) to an externally mounted formaldehyde reservoir (1) positioned on top of the apparatus. The refilling valve (7) was controlled by a 61F-GP-V50 floatless level switch (3) (O.T.E. Japan) whose sensing probes (5) were positioned inside the glass syringe barrel (4). The floatless level switch was in turn controlled by the formaldehyde entry delay timer (2). The timer was set at 6 seconds, the lag period between the injection of formaldehyde into the chamber and the admission of steam. At the time when formaldehyde was required, i.e. after the initial steam pulses, the sequential controller would move to a position to energise the delay timer and the formaldehyde entry solenoid valve. The set volume of formaldehyde would then be discharged gravimetrically from the syringe barrel to the vapouriser and subsequently into the chamber. During the time the delay timer was still activated, the floatless level switch would be activated and in turn energise the formaldehyde refilling solenoid valve. Formaldehyde would then flow gravimetrically from the reservoir to the syringe barrel, to the level determined by the distance between the tips of the sensing probes. The amount of dispensed formaldehyde solution was determined by altering the distance between the sensing probe tips. The delay timer had the dual role of controlling the refilling time and also of controlling the lag period between formaldehyde and steam injection at the start of the hold period. The delay timer was set at 6 seconds such that the lag

period was greater than the time required to empty the set volume but less than the sum of the time required to empty the whole syringe contents and refill it.

A leak test was performed following the installation of the formaldehyde input and measuring device to the machine after the addition of the sampling system (see Section 6.2.2). This was performed by allowing the machine to pull a vacuum of about 800 mbar gauge, just before the initiation of the first steam pulse. The mains were switched off and supplies to the vacuum pump and cooling fans cut. The loss of vacuum, caused by the leaking in of atmospheric air, was then determined over 10 minutes. A leak rate of 20 mbar gauge over a period of 10 minutes was recorded. The installation of the device did not constitute any additional loss of vacuum since the leak rate obtained was the same as that observed prior to the installation (see Table 19).

6.2.1.5 Positioning of the Apparatus

A trial LTSF cycle of the modified apparatus revealed condensation of steam on the unheated door interior and subsequent collection of condensate in the sampling ports (Section 6.2.2). The condensate would affect the concentration of the gaseous formaldehyde in the chamber and was expected to interfere with sample retrieval. The problem was overcome by positioning the apparatus on a mobile stage resting at an angle of about 30° to the horizontal. Any condensate would then be instantly drained out via

the chamber drain.

6.2.2 Samping System

To enable the bacterial test pieces to be exposed to LTSF conditions a sample insertion and retrieval device had to be designed and fitted to the door of the machine. Five sampling ports, each incorporating a manually operated ¾" ball valve (CMI, Ascot, Berkshire) were inserted into the steel door. The proximal end of the sampling port was fitted with a short length of ¼" O.D. copper tube which was connected to a brass T-section. One arm of the T-section was connected to the atmosphere through a ¼" ball valve and a 13 mm diameter "Swinnex" unit with a 0.2 µm cellulose acetate membrane filter (Sartorius Instruments). The other arm of the T-section was connected to the vacuum line via a ¼" ball valve (Fig. 36). The vacuum and air lines were necessary to allow sample insertion and retrieval during an operational cycle without disturbing chamber conditions and also without the risk of physical loss of spores from carriers due to the changes in pressures between the outside and inside of the chamber.

A secondary brass chamber was constructed such that it screwed axially onto the sampling port against a rubber "O"-ring. A steel operating rod (430 mm) was included in the secondary chamber such that it could be moved in and out of the main chamber through the opened ¾" ball valves of the sampling ports. The distal end of the operating rod allowed for the test piece carrier to be threaded

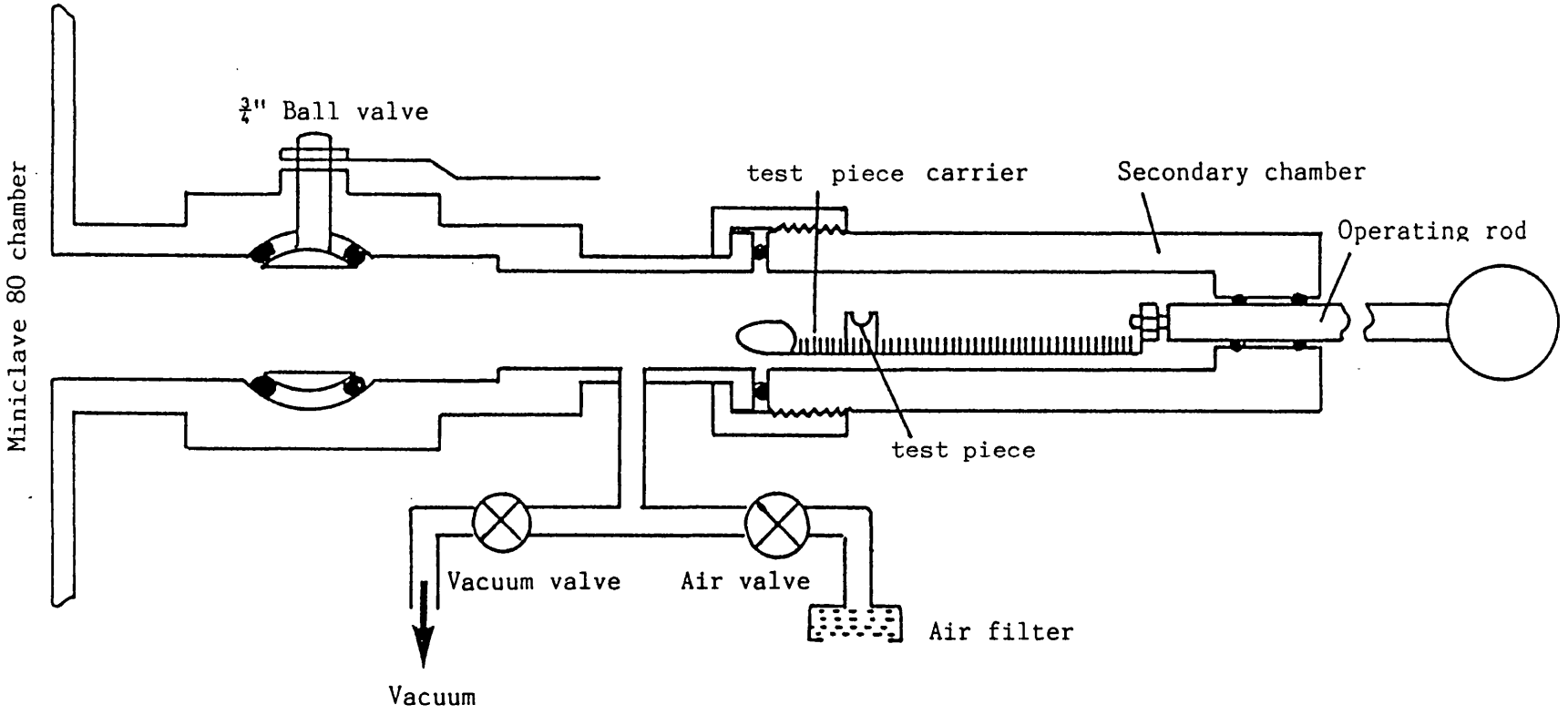


Figure 36. Diagram of the sampling system. From Line, S.J. (229)

onto it. The test piece carrier comprised of a 50 mm thin wire spring soldered onto a stiff wire to hold it in place and to support its length. At one end of the wire support was attached a 6BA nut which screwed onto a short length of 6BA threaded stub, attached at the end of the operating rod (Fig. 36). Each test piece carrier could hold up to five test pieces allowing quintuplicate determinations of survivors for each of the sampling intervals.

A leak test was performed as determined in Section 6.2.1.4 to check if the modifications to the door described here, affected the leak rate of the machine. Data displayed in Table 19 show the leak rate of the unmodified apparatus, for the apparatus with the ¾" ball valve of the sampling port closed, for the apparatus with the ¾" ball valve open and operating rods inserted into the main chamber and finally after installation of the formaldehyde input and measuring device (Section 6.2.1.4).

The results in Table 19 show that the insertion of the five sampling ports onto the door of the machine did not significantly affect the leak rate of the original design. However, when the ¾" ball valves were open with the secondary chamber attached and operating rods inserted into the main chamber, a 33.33% increase in the leak rate was observed. This was to be expected. The operating rods, sealed with two "O"-rings at both ends of the secondary chamber, were fitted such that they could be moved in and out of the chamber without any force. Sideways motion, though unintended, was possible at the seals, so an air tight seal could not be

achieved. It was considered that this leak rate was acceptable and unlikely to affect the operation of the machine.

Table 19. Data Displaying the Leak Rates of the Miniclave 80 Before and After Modifications to the Door and After Installation of the Formaldehyde Input and Measuring Device

State of Machine	Leak Rate Determined Over 10 Minutes
Original Machine	15 mbar
Modified Door ¾" Ball Valves closed	16 mbar
Modified Door ¾" Ball Valves open and Operating Rods inserted into Main Chamber	20 mbar
¾" Ball Valves open, operating rods inserted and Formaldehyde input and measuring device installed	20 mbar

Operating temperature = 73° ± 0.5°C

The pipework diagram of the modified Miniclave 80 with the sampling system, the steam supply and the formaldehyde input and measuring device is illustrated in Figure 37.

6.2.3 Operation of the Modified Miniclave 80

The operation of the modified machine for experimental purposes was as sequenced below.

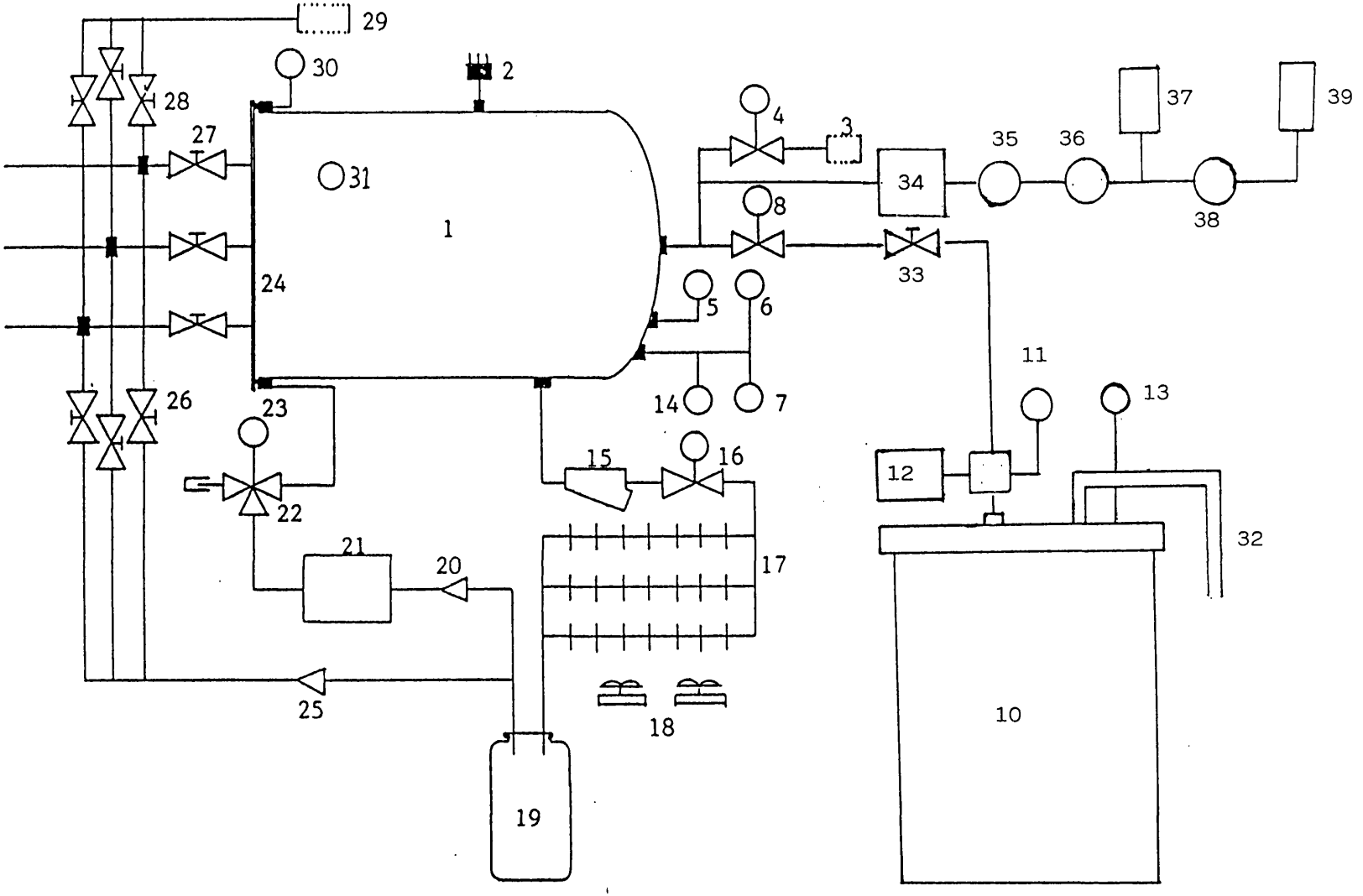
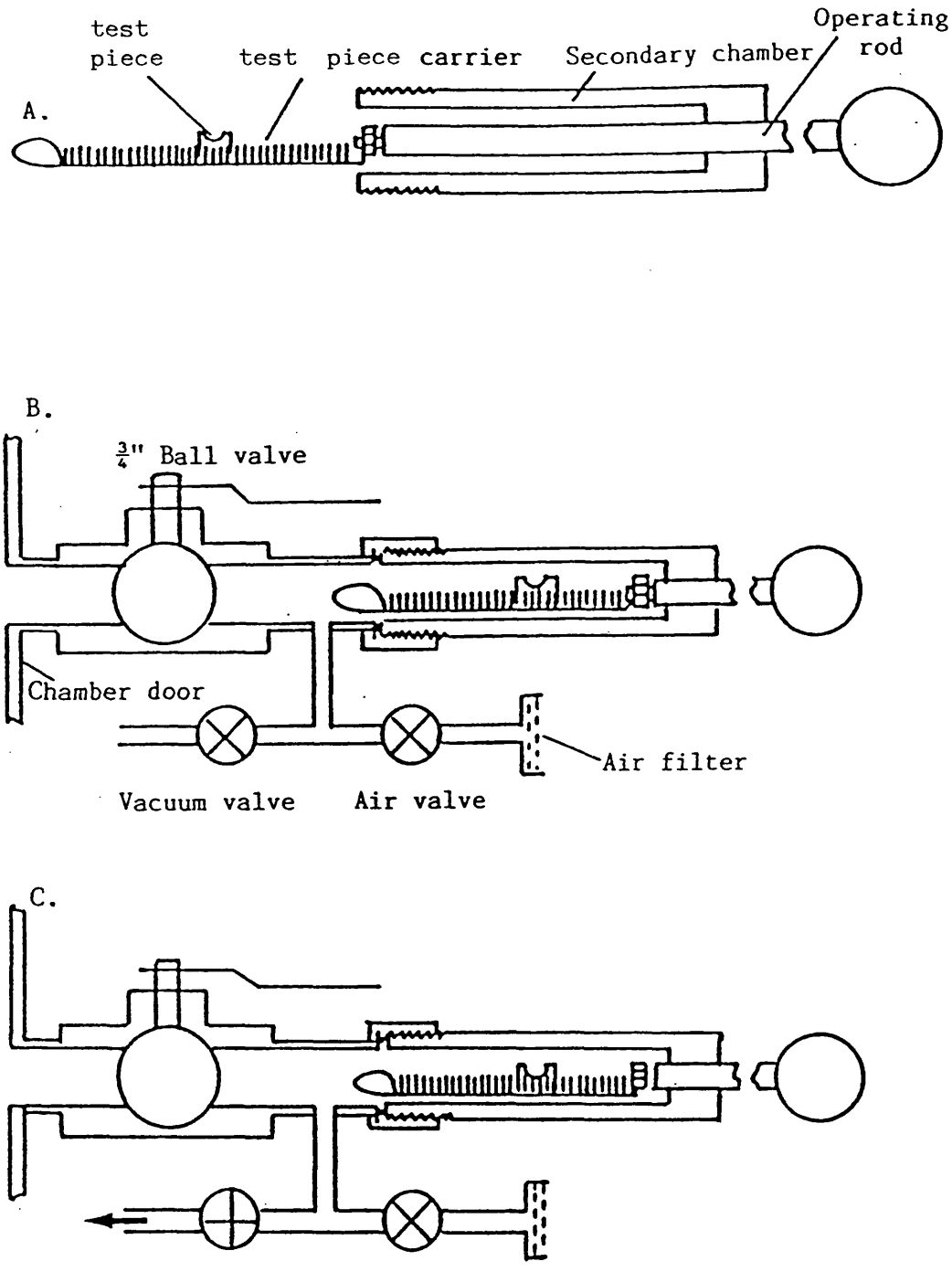


Figure 37. Pipework diagram of the Miniclave 80 chamber, steam generator, sampling system and formaldehyde input and measuring device. Developed from a pipework diagram prepared by Line, S.J. (229)

Key to Figure 37. Pipework Diagram of the Miniclave 80 Sampling System Modified Steam Generator and Formaldehyde

1. Miniclave chamber with electric jacket heating.
2. Thermocouple entry port.
3. Air filter.
4. Air entry solenoid control valve.
5. Temperature cut out and thermostat switches.
6. Vacuum gauge.
7. Temperature gauge.
8. Steam inlet solenoid valve.
9. Steam inlet flow control valve.
10. Electrically heated steam generator.
11. Pressure safety switch.
12. Pressurestat.
13. Pressure/temperature gauge.
14. Vacuum switches.
15. Strainer.
16. Drain solenoid valve.
17. Heat exchanger.
18. Cooling fans.
19. Vacuum trap bottle.
20. Non return valve.
21. Vacuum pump.
22. Three way exhaust valve.
23. Door seal pressure system.
24. Miniclave door.
25. Nonreturn valve.
26. Vacuum control valves for door sampling system.
27. Door control valves for door sampling system.
28. Air control valves for door sampling system.
29. Air filter.
30. Door seal pressure switch.
31. Chamber over temperature thermostat.
32. Bleed pipe.
33. Steam inlet flow control valve.
34. Vaporiser.
35. Check valve.
36. Formaldehyde entry solenoid valve.
37. Formaldehyde measuring cylinder.
38. Formaldehyde refilling solenoid valve.
39. Formaldehyde reservoir.

1. Before the commencement of an LTSF cycle, it was necessary to check and ensure that:
   a) The vacuum trap bottle was empty;
   b) the steam generator contained sufficient distilled water, at least 5 litres;
   c) the formaldehyde reservoir was full and the measuring and input device correctly set;
   d) the jacket temperature was set at 2°C above the chosen operation temperature;
   e) the key switch was in the Automatic mode and the "hold period" timer correctly set;
   f) the CAL-9000 temperature control was correctly set to the desired operation temperature.
2. The door seal "O" ring was smeared with vacuum grease, the door closed and the machine started with all valves shut.
3. The machine was allowed to proceed through the initial evacuation of the chamber and the five steam and vacuum pulse stage.
4. While the machine was steam pulsing, test pieces were aseptically secured onto the test piece holders at the end of the operating rods (Fig. 38A). The loaded test piece carriers were retracted into the secondary chambers which were then secured onto the ¾" ball valves of the sampling ports ((Fig. 38B).
5. Each of the ¼" ball valves on the secondary chambers was opened momentarily and shut to equilibrate the pressures in the sampling ports and the secondary chamber (Fig. 38C).



(From Line, S.J., (229))

Figure 38. Sampling system operating sequence.

A. Test piece holding device loaded with a test piece.

B. Test piece holding device withdrawn into secondary chamber and chamber screwed onto ¾" ball valve. All valves closed.

C. Vacuum valve opened to evacuate the secondary chamber.

Figure 38. Sampling system operating sequence.

D. Vacuum valve 'j' closed, ¾" ball valve opened and timer started.

E. Test piece holder with test piece inserted into chamber.

F. On completion of the exposure time test piece holder withdrawn from chamber, ¾" ball valve closed, air admission valve opened, and secondary chamber removed from ¾" ball valve and test piece removed.

From Line, S.J., (229)

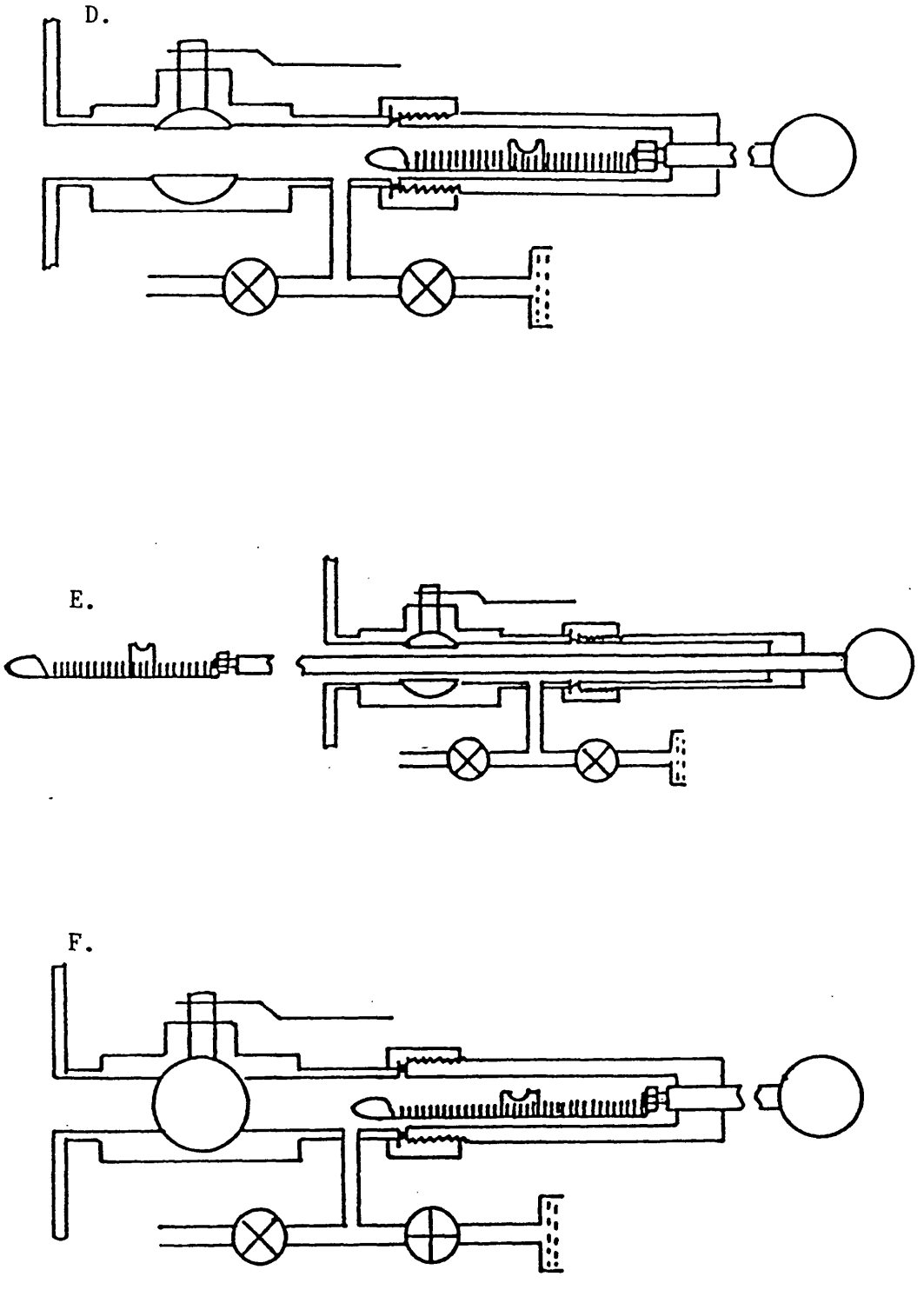


Figure 38. Sampling system operating sequence

6. As soon as formaldehyde solution had been dispensed into the vaporizer and the chamber temperature had been reached, the ¾" ball valves were opened to expose the samples to chamber conditions. This marked the beginning of the exposure period. The operating rods were then pushed fully into the chamber to expose samples in the centre of the chamber (Fig. 38D-E).

7. At predetermined time intervals within the hold period, each operating rod and samples was retracted from the chamber and the ¾" ball valve shut. The air vent valve was then opened to equilibrate pressures within the secondary chamber and the atmosphere (Fig. 38F). The secondary chamber was then unscrewed from the sampling port and samples aseptically transferred into sterile inactivator. Survivors were then recovered as described in Section 6.3.2.

6.2.4 Preparation of Test Pieces

6.2.4.1 Preparation of Carriers

Carriers suitable for use with the sampling system described above were prepared from 20 mm x 13 mm aluminium foil strips with a 6 mm diameter well in the centre (Albert Browne Ltd., Leicester). These were cut to approximately 15 mm x 8 mm and the ends bent at right angles to provide mountings to fit into the test piece holder (Fig. 39).

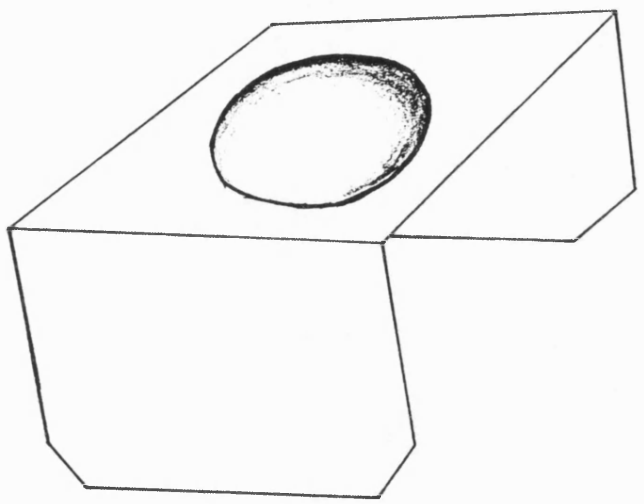


Figure 39. Diagram of the Aluminium Carrier.

The carriers when then soaked overnight in a dilute solution of Linkdet 704 (Link Chemicals Ltd.) followed by five washes in running tap water and five rinses in glass distilled water. The cleaned carriers were then immersed in acetone for three hours with occasional shaking before being allowed to dry covered in clean glass petri dishes. Sterilization was by hot air at 160°C for at least 60 minutes.

6.2.4.2 Loading of Carriers

The sterile aluminium carriers were aseptically mounted by pressing the legs into sterile 1% plain Oxoid No 3 agar (Lab-M Ltd.) under laminar flow conditions. Appropriate aliquots of spore suspensions containing approximately 1×10^6 spores were distributed into the carrier wells with vigorous shaking of stock suspension between each aliquot. The preparations were left to dry under laminar flow conditions for six hours at 25° ± 2°C and used on the day of preparation within an hour after the preparations were dry.

6.3 EXPERIMENTAL

6.3.1 Determination of Viable Count of Spores Recovered from Aluminium Carriers

In order for the test pieces to be of use in assessing the sporicidal activity of LTSF it was necessary to demonstrate that the spores could be reproducibly removed from aluminium carrier surfaces without significant loss of viability.

Loaded carriers, each containing approximately 1×10^6 spores were aseptically transferred into 10 ml sterile glass distilled water in thin-walled 30 ml glass screw-capped bottles (Aimer Products Ltd., London). The containers were then suspended loosely from racks which allowed their bases to lie at least 30 mm from the surface of the fluid in the ultrasonic bath (Bransonic Ultrasonic Cleaner, USA). The racks were positioned such that the containers would be about 80 mm from the short sides of the bath. The ultrasonic bath was filled with glass distilled water containing 0.3% v/v Tween 80 ensuring the level in the bath was above sample levels in the containers. The samples were then subjected to ultrasonic treatments for five minutes at 51 KHz ± 6% oscillation frequency. Electronmicroscopic examination of sonicated test pieces revealed that most spores were detached from carrier surfaces after 2 mins of sonication (Plate 1). Serial dilutions (0.5 + 4.5 ml) were performed in sterile distilled water and the viable count of the samples determined by the spread plate

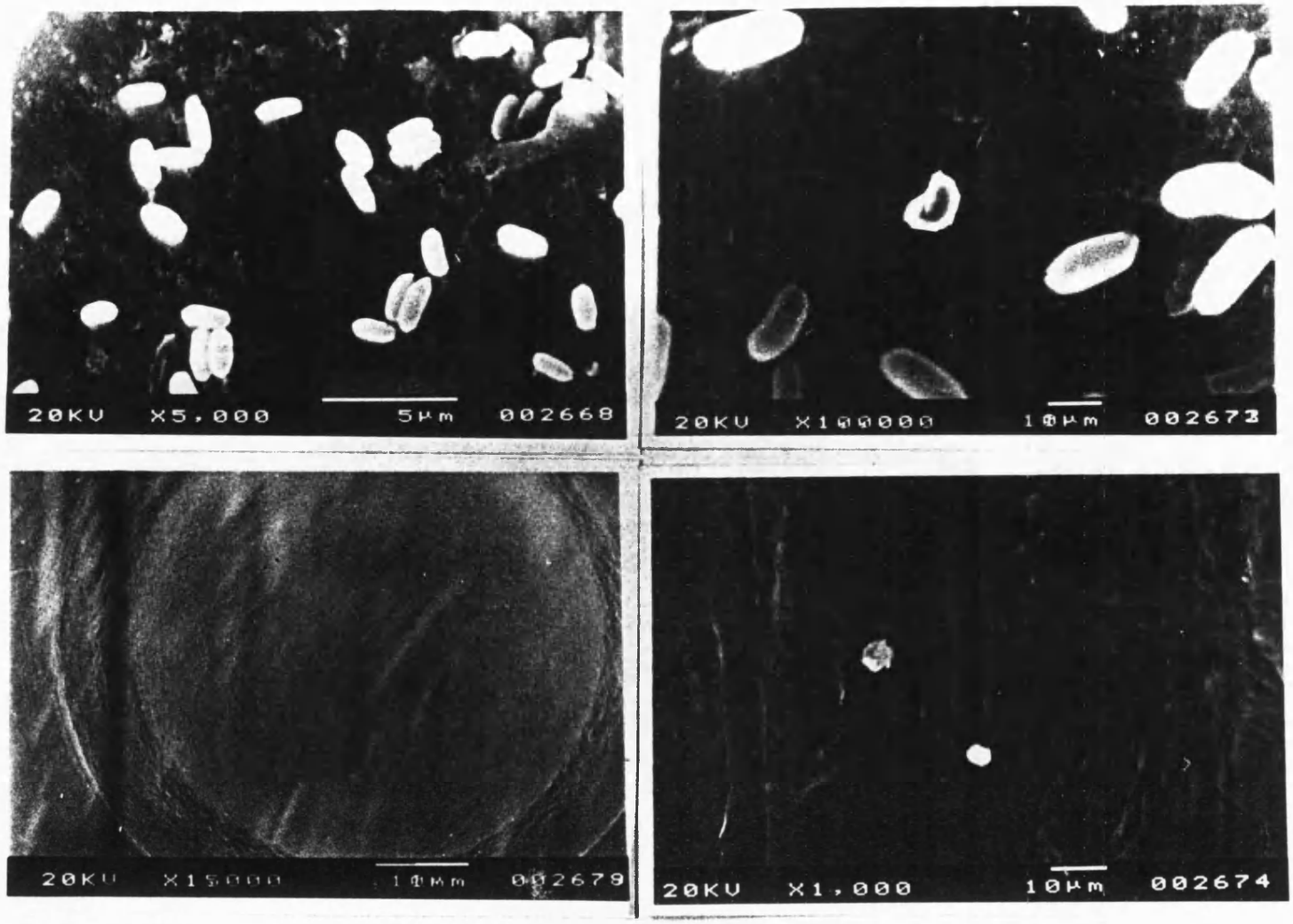


PLATE I. ELECTRON MICROGRAPHS OF ALUMINIUM CARRIER SURFACES AFTER DIFFERENT ULTRASONICATION PERIODS AT 51 KHz. OF TEST PIECES OF BACILLUS STEAROTHERMOPHILUS NCIB 8224 SPORES. CLOCKWISE FROM No.002668 After 0 mins; After 1 min; After 2mins and after 5 mins.

technique using five replicate recovery plates for each sample.

Ten test pieces were randomly selected from a population of 40 and the viable count from each of the test pieces determined as described above. Spores were recovered on NA medium after incubation at 56°C for 48 hrs. Data displayed in Table 20 show the reproducibility of recovering spores of B. stearothermophilus NCIB 8224 Batch (B15F) produced on C-Ltd medium and the Analysis of Variance of this data. The original spore suspension contained 5.2 x 10^7 spores/ml and thus the viable count administered on carriers (contained in 20 µl) was 1.05 x 10^6 spores. A mean viable count of 9.46 x 10^5 spores per carrier was obtained and this represented a 90.1% recovery of the number of viable spores inoculated onto the carriers.

The variation observed in the number of spores recovered between the test pieces was significantly greater than the variation observed within replicates of the samples. However, since the overall coefficient of variation, 0.7%, is within statistically acceptable limits, the technique can be considered suitable for the recovery of spores from carrier surfaces.

6.3.2 Effect of the Initial Vacuum and Steam Pulses in the Miniclave 80 Cycle on Test Pieces

In practice, biological indicators are usually included in the load to be sterilized and will therefore be subjected to the

Table 20. Data showing Reproducibility of Recovery of Spores from Aluminium Carriers using Ten Replicate Test Pieces of B. stearothermophilus NCIB 8224 Produced on C-Ltd Medium

Test Piece	Dilution Plated	Plate Counts	Mean	Mean Viable count recovered (in 10 ml)
1		185,184,186,187,185		
2		185,185,187,187,186		
3		191,194,190,190,193		
4	10^2	192,191,194,193,192	189.2	9.46×10^5
5		189,188,189,190,189		
6		194,192,189,191,193		
7		185,187,188,189,189		
8		192,191,190,190,194		
9		188,184,185,185,186		
10		192,190,191,191,192		

Sources of Variance	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio (F)
Between Replicates	9	359.2	39.911	20.8
Within Replicates	4	9.6	2.4	1.2
Residual	36	69.2	1.9222	
Total	49	438		

Coefficient of Variation (C.V.) = 0.7%

$F(4, 36)\ P_{0.05} = 2.61$

$F(9, 36)\ P_{0.05} = 2.12$

initial vacuum and steam pulses of the LTSF cycle. The recovery of spores at the end of the cycle could reveal much less viability and imply exaggerated efficiency of the cycle if the physical effect of the vacuum and steam pulses on the test pieces is not considered. Usually test pieces are presented to the sterilizers within primary packs which have a defined permeability to the sterilizing agent(s) of the process in which they are to be used. The primary pack would theoretically enable any spores that might have suffered physical stress of the vacuum and steam pulses to be recovered. However, in practice, the test pieces are invariably aseptically removed from the primary packs and inoculated into recovery media, and thus spores that are physically removed from test pieces are not recovered. In view of this, it was considered necessary to investigate the effect of the vacuum and steam pulses in the Miniclave 80 cycle on the viability of spores administered on the aluminium carriers.

Spores of B. stearothermophilus NCIB 8224 (B15F) produced on C-Ltd medium were used to prepare test pieces as described in Section 6.2.4.2. Five test pieces were secured on each of the test piece holders of the operating rods and inserted into the chamber of the Miniclave 80. The machine, set to operate at 73°C, was started and left to cycle through its automatic LTSF cycle mode. Each test piece holder was retrieved from the chamber immediately after each steam and vacuum pulse using the procedure outlined in Section 6.2.3. The viable count on each test piece was then determined using the method described in Section 6.3.1. Spores were

recovered on NA plates incubated at 56°C for 48 hrs. The mean viable count of spores recovered after each pulse was determined and the results recorded in Table 21. Data in Table 21 also show the fraction of the initial numbers administered (8.7×10^5 spores) that were recovered after each steam and vacuum pulse expressed as a percentage. Figure 40 shows the plot of the recovered fraction as a percentage, on a logarithmic scale, against the corresponding time in minutes of each sample, on a linear scale. It is evident from the data displayed in Table 21 and Fig. 40 that there is a progressive decline of the number of viable spores recovered from the test pieces with each additional steam and vacuum pulse. The results show that after five successive steam and vacuum pulses of the standard Miniclave 80 cycle, the percentage of the viable spores recovered from the test pieces was reduced to 40.2% of the initial load. This loss in viable count on test pieces during this initial stage of the LTSF cycle was attributed to a physical loss rather than an inactivation by saturated steam because during this stage, the machine would be heating up to the set sterilizing temperatures and temperatures below 80°C are not expected to have any significant lethality on spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium.

The total count of spores remaining on the carriers could have been determined to check if the effect of the steam and vacuum pulses was a physical removal rather than an inactivation by saturated steam at temperatures below 80°C. However, this was not practical in this investigation since the spore numbers

Table 21. Data Showing the Effect of Steam and Vacuum Pulses in the Miniclave 80 Cycle on the Viable Count of B. stearothermophilus NCIB 8224 spores on test pieces

Pulse	Sampling Time (mins)	Mean Viable Count Recovered from Pulsed Test Pieces	% Nt/No
1	3.45	6.1×10^5	70.1
2	4.65	5.3×10^5	60.9
3	5.70	4.4×10^5	50.6
4	6.75	3.6×10^5	41.4
5	7.80	3.5×10^5	40.2

Initial viable count administered on test pieces = 8.70×10^5 spores

administered would be too low to be accurately counted in a counting chamber.

The effect of equilibrating secondary chamber pressure and main chamber pressure prior to the insertion of the operating rods into the chamber (Section 6.2.3), was determined on the test pieces of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium. Five test pieces each containing 8.7×10^5 spores were subjected to the equilibration treatment and the viable count of spores recovered from each test piece determined. A mean viable count of 8.48×10^5 spores was obtained, i.e. 97.5% of the initial

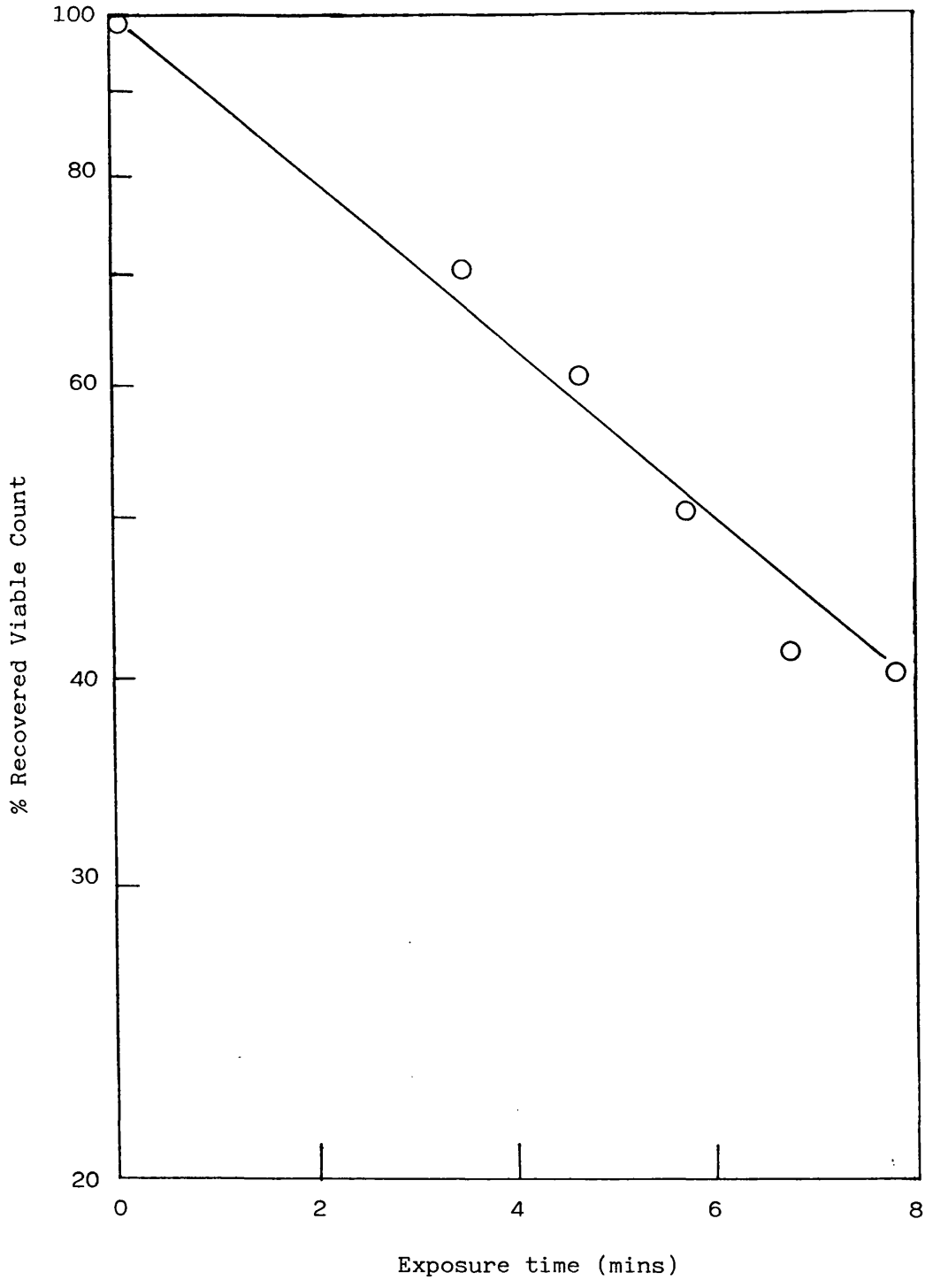


Figure 40. The effect of steam and vacuum pulses of the modified Miniclave 80 LTSF cycle at 73°C on the viable count of test pieces of B. stearothermophilus NCIB 8224 produced on C-Ltd medium.

load. Equilibrating secondary and main chamber pressures reduced the viable count administered on test pieces by 2.5%.

Subsequent experiments to be reported in this thesis were carried out to determine the response of bacterial spores when exposed to various LTSF conditions. Therefore, in the light of the effect of the steam and vacuum pulses on the test pieces investigated above, test pieces will be introduced to the chamber atmosphere after the initial steam and vacuum pulse stage of the modified Miniclave 80 cycle. Equilibrating secondary and main chamber pressures did not have as much effect as the steam and vacuum pulses. Therefore the time zero (100%) samples will be subjected to the equilibration treatment to standardize the inactivation treatments with the other test samples.

6.3.3 Choice of Formaldehyde Inactivator for LTSF Studies

For quantitative evaluation of the sporicidal properties of LTSF, an inactivator had to be used to terminate the lethality of any residual formaldehyde present on the spores at the time of sampling. A 10% w/v glycine solution was used as inactivator during the determination of spore resistance to 0.5% w/v formaldehyde in aqueous solution (Chapter 5). Hoxey (22) has shown that while this concentration is effective as an inhibitor of formaldehyde at concentrations up to 30% w/v in aqueous solution, its presence either in the suspension that is plated out or in the recovery medium, can be inhibitory to the germination and outgrowth of some

spores. This situation could arise at low survivor levels where samples are plated out directly from the inactivator solution. In previous experiments, a membrane filtration method was used for recovery of low numbers of survivors, to overcome this problem (Chapter 5). To obviate the need for filtration, a 1% w/v strength was used to inactivate formaldehyde following the exposure of spores to LTSF conditions. 1% w/v glycine solution has been used to inactivate 0.5% w/v formaldehyde in aqueous solution without any effect of recovery of survivors when transferred to the recovery medium (74). A comparison of viable counts obtained using sterile distilled water as diluent and with 1% w/v glycine solution as the first of a ten fold serial dilution, was performed and the results recorded in Table 22. A two sample 't' test was carried out on the data displayed in Table 22 to assess comparability between the two treatments. The results of the t-test show that there is no significant difference between the two treatments. Therefore the use of 1% w/v glycine solution as the first of a ten-fold serial dilution did not affect the germination and outgrowth of the bacterial spores on nutrient medium.

Therefore in subsequent experiments, each test piece after exposure to LTSF, was aseptically transferred to 10 ml of sterile 1% w/v glycine solution and subjected to ultrasonic treatment for 5 mins. Surviving organisms were then recovered as described in Section 6.3.1.

Table 22. Comparison of Viable Count Obtained Using (a) Sterile Water and (b) Sterile 1% w/v Glycine Solution as the First Diluent in a Serial Dilution

(a) Sterile Water

Sample	Dilution	Plate Counts	Mean	Mean Viable count spores/ml
1		150,153,150,149,152		
2	5×10^3	147,145,143,158,147	150.8	3.77×10^6
3		150,152,149,148,157		
4		149,150,152,154,150		
5		153,151,152,155,154		

Coefficient of Variation (C.V.) = 2.29%

(b) 1% w/v glycine in sterile water

Sample	Dilution	Plate Counts	Mean	Mean Viable Count Spores/ml
1		147,153,154,149,158		
2	5×10^3	157,156,149,150,152	152.24	3.81×10^6
3		149,147,140,143,146		
4		161,160,159,160,160		
5		152,158,147,149,150		

Coefficient of Variation (C.V.) = 3.84%

$t = \frac{\bar{x} - \bar{y}}{SE(\bar{x}-\bar{y})} = 1.06$ where $\bar{x}$ = mean colony count from (a)
$\bar{y}$ = mean colony count from (b)
SE = standard error

Tabulated t value for 48 degrees of freedom = 2.02 at 0.05% confidence interval

6.3.4 Exposure of Spores of B. stearothermophilus NCIB 8224 Produced on C-Ltd Medium to LTSF at 80°C and 16.38 mg l^{-1} Formaldehyde Concentration in the Modified Miniclave 80

For the initial studies on the effect of LTSF on bacterial spores, the conditions used were those considered to be the most extreme that were likely to be tested using the modified Miniclave 80. This was a concentration of 16.38 mg l^{-1} formaldehyde (equivalent to injecting 1 ml 38% w/v formaldehyde solution into the Miniclave 80 chamber of internal volume 0.0232 m^3) and a temperature of 80°C ± 0.5°C. With the apparatus set to operate under these conditions, test pieces containing approximately 10^6 spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium were exposed to the 'hold' conditions of the LTSF cycle using the procedure outlined in Section 6.2.3.

Samples were withdrawn at predetermined time intervals from the chamber atmosphere over a period of 2 hours and the number of surviving organisms determined as described in Section 6.3.3. The data displayed in Fig. 41 show the surviving fractions as a percentage plotted on a log scale as a function of the exposure time for each of the three replicate determinations. These data reveal significant variation between the triplicate determinations of the response of the test spores to inactivation by LTSF at the defined conditions. This variation is evident from the wide scatter of survival data observed in Fig. 41. A mean survivor curve was constructed through these data and consisted of two distinct linear

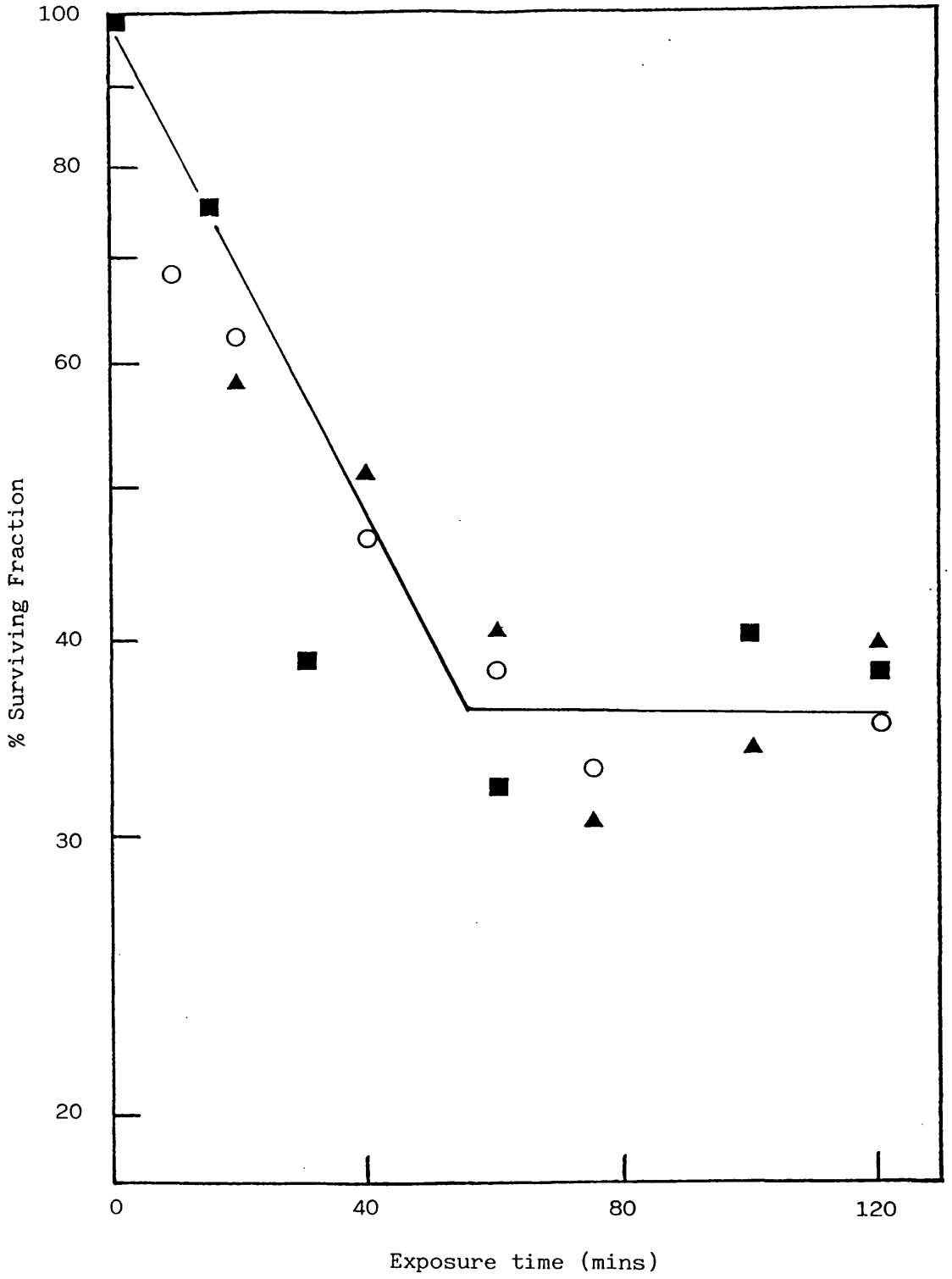


Figure 41. Triplicate determination of response of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium to exposure at 80°C; 16.38 mg l^{-1} formaldehyde concentration.

phases. Exposure of the test pieces to periods greater than 60 minutes did not cause any significant reduction in survivor levels. Furthermore, the observed variation in response between the triplicate determinations was greater for exposure periods in excess of 60 minutes.

6.4 DISCUSSION

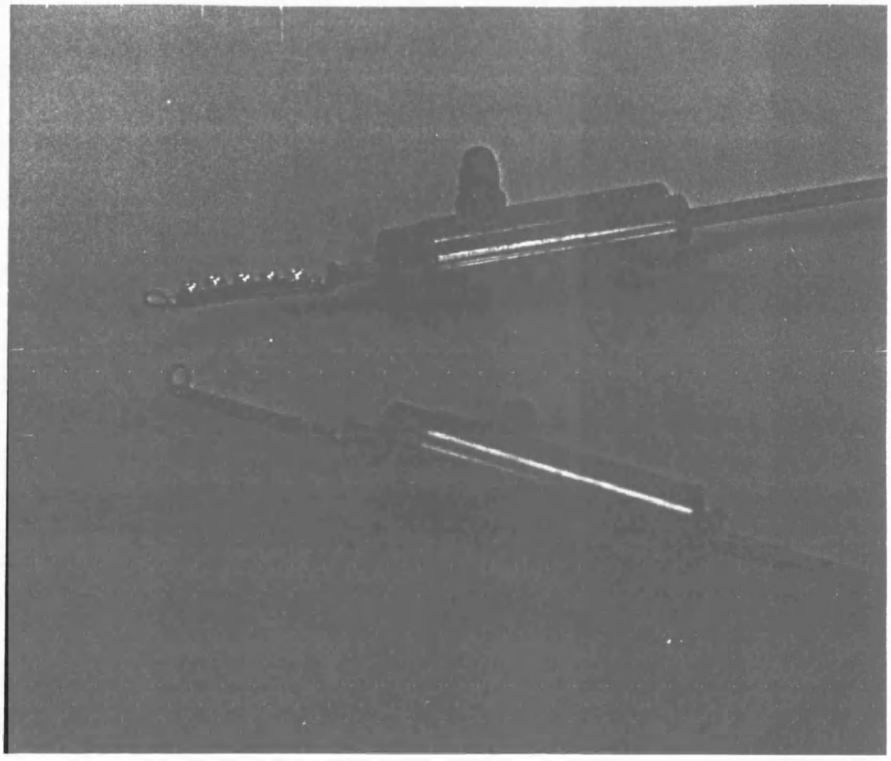
The Miniclave 80 LTS/LTSF sterilizer was selected for modification to a standard experimental apparatus since it was the only commercially available small unit able to provide both LTS and LTSF cycles. The original design was inadequate for carrying out experimental investigations. The modifications described in this chapter were undertaken to offer more flexibility in the use of the apparatus to allow parameters of LTS and LTSF to be varied in a defined and reproducible manner.

An external steam supply was fitted to provide steady saturated steam for prolonged periods and the avoidance of the use of the original vapouriser to generate steam minimised the risks of creating superheat conditions in the main chamber. To provide easy and accurate adjustment of the operating temperature, an adjustable electronic temperature controller (CAL-9000) was substituted for the original pressure control. This maintained the set temperature within a variation of ±0.5°C. The jacket temperature was set at 2°C above the defined operation temperature to avoid creation of superheat conditions. The apparatus comprised of a fail safe

mechanism which would disrupt the cycle if the jacket temperature was not set correctly.

For the purpose of this investigation, it was necessary to vary the concentration of formaldehyde in the chamber. A formaldehyde input and measuring device was designed and fitted to the machine. This device could be adjusted to deliver 0.5 ml to 3.0 ml of 38% w/v formaldehyde solution (with 10% methanol as stabilizer) equivalent to 8.19 mg l^{-1} to 49.14 mg l^{-1} gaseous formaldehyde in the chamber. This is assuming of course that all the formaldehyde delivered was fully vaporized and admitted to the chamber.

A sampling system was necessary to enable the insertion and retrieval of samples during the LTSF cycle without disturbing the chamber conditions. The sampling system described in this chapter was reliable and quick to operate and did not necessitate an allowance for warm-up time. The design of the sampling system was such that when test pieces were introduced to the chamber they would be positioned in the central region, thereby minimising any influences on the test pieces that could have been caused by possible variation in conditions within the chamber. The design allowed for the exposure of samples to the chamber conditions for a minimum exposure period of 1 minute. Plate 2 displays the secondary chambers with the operating rods, the test piece carriers and the door sampling port assembly.



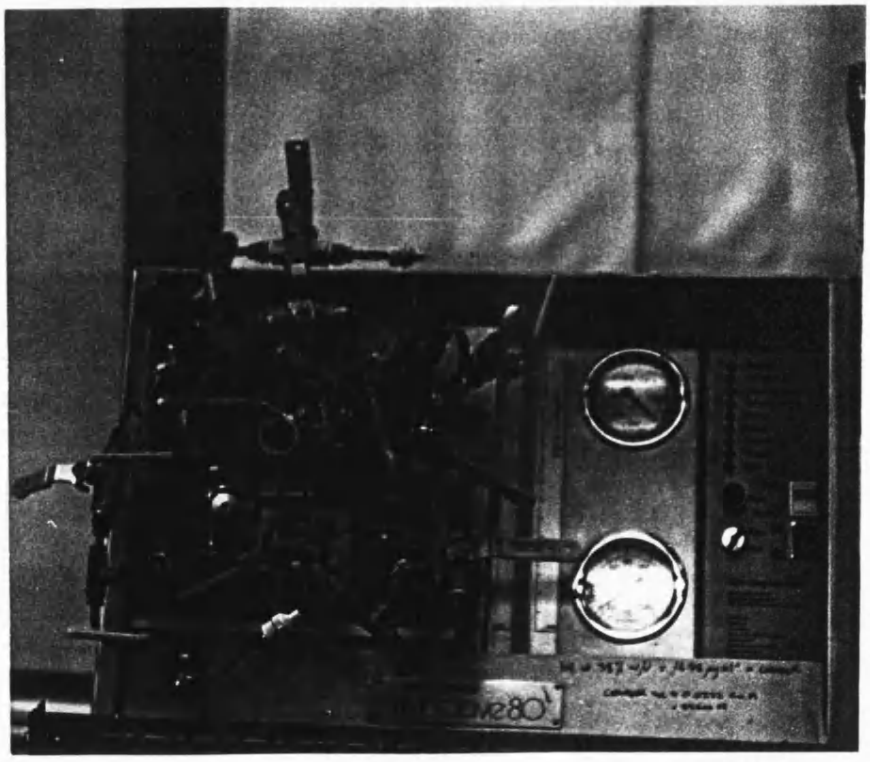


PLATE 2. Photographs showing (Top), Secondary chambers with test piece holders, with or without the test pieces and (Below), the Front panel of modified Miniclave 80 displaying the door sampling port assembly.

The original Miniclave 80 had an inherent leak rate of 15 mbar over 10 minutes. Modifications to the door (for the sampling system), installation of the formaldehyde input and measuring device and substitution of the steam generation system increased the leak rate to 20 mbar over 10 minutes. This was considered unlikely to affect the control and operation of the machine as the chamber is under constant vacuum during operation. Marcos and Wiseman (131) did not consider increased leak rates following modifications on a similar machine to have significant effect on the operation of the machine.

The protocol developed for the preparation of carriers and test pieces was intended to standardize pretreatment conditions on the test pieces to achieve good reproducibility within and between batches of test pieces. Relative humidity can significantly influence resistance of bacterial spores to inactivation by LTSF conditions (131, 133). A standardized method of preparation of test pieces, under laminar flow at room temperature guaranteed uniformity in those variants that would otherwise influence spore resistance.

The accuracy of estimating surviving organisms after LTSF treatment depends on the efficiency of removing treated spores from the carrier surface and the subsequent ability to recover and count the surviving organism. Ultrasonication of the test pieces showed that the technique was reproducible in detaching spores from carriers. In this case, a 90.1% recovery of the bioburden on the

test pieces was achieved after 5 minutes treatment at 51 kHz. Burgos et al. (230) demonstrated that ultrasonic treatment did not have any significant effect on the viable count of treated or untreated spores of B. cereus and B. licheniformis. However, ultrasonic treatment was reported by the same workers to decrease subsequent heat resistance of the spores (230).

Sterile 1% w/v glycine solution was used to inactivate any residual formaldehyde at the time of sampling. This concentration has been demonstrated to have no inhibition on the germination of untreated spores when transferred to the recovery medium (Section 6.3.3).

Steam and vacuum pulses necessary for humidifying the chamber and to heat the apparatus to the set temperature had a significant effect on viable count administered on aluminium carriers. Five steam and vacuum pulses reduced the viable count on aluminium carriers to 40.2% of the original bioburden. In subsequent experiments, test pieces were introduced into the chamber after the initial pulsing stage since the study was principally concerned with the effect of 'hold' conditions of LTSF on bacterial spores. In process validation however, biological indicators (i.e. a test piece contained within its primary pack ready for use in the relevant sterilization process without further modification) have to be subjected to the same treatment experienced by the load and therefore have to be introduced to the chamber prior to the initial pulsing stage. Any spores detached by

the effect of the pulses will be contained within the primary pack and thus biological indicators are only sufficient for qualitative process monitoring.

Equilibrating secondary and main chamber pressures before insertion of samples to the chamber has been shown to reduce the bioburden on the test pieces by 2.5%. This procedure was necessary to avoid significant physical removal of spores from carrier surfaces. Therefore the time zero (100%) samples had to be subjected to similar equilibration treatment.

Spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium have been recommended, from characterisation studies reported in this thesis, as suitable for development as biological indicators for LTSF sterilization. Spores of this organism were therefore selected to evaluate the performance of the modified Miniclave 80 LTSF cycle. A bi-phasic survivor curve was produced from the data obtained from triplicate determination of response to LTSF at 80°C, and 16.38 mg l^{-1} gaseous formaldehyde. In all three replicate determinations of the response to LTSF at the set conditions, the initial exponential decline was linear for much less than one log cycle. Cerf (231) suggested that a mixed age spore suspension could be expected to produce such inactivation kinetics when spores cultured from the more resistant survivors would be more resistant than the original population. Spores used in this evaluation were expected to be of the same age and inactivation kinetics implying mixed age spore suspension were not

observed when spores of this strain were exposed to heat at 110°C and to 0.5% w/v formaldehyde in aqueous solution at temperatures between 60° and 80°C. A normal distribution of resistance in the spore suspension would be expected but then a gradual decrease (Type C) in the death rate would be expected where spores cultured from the more resistant survivors do not exhibit greater resistance than the original population (7).

A change in the exposure conditions, in this case a reduced lethality of the process due to likely changes in the availability of formaldehyde could provide an explanation for this phenomenon.

CHAPTER 7

EFFECT OF TEMPERATURE AND FORMALDEHYDE CONCENTRATION ON THE INACTIVATION BY LTSF OF *BACILLUS STEAROTHERMOPHILUS* NCIB 8224 SPORES PRODUCED ON C-LTD MEDIUM

7.1 INTRODUCTION

When spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium were exposed to LTSF at 80°C at a formaldehyde concentration of 16.38 mg l^{-1}, a bi-phasic survivor curve was obtained (Section 6.3.4). Prolonged exposure at these LTSF conditions did not produce any significant decline in the number of surviving spores. It was necessary to establish whether bi-phasic inactivation kinetics occurred over the range of temperature and formaldehyde concentrations that have been employed in commercial LTSF sterilization.

The present chapter describes experiments carried out to investigate the effect of temperature and formaldehyde concentrations on the resistance of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium, to inactivation by LTSF using the modified Miniclave 80.

7.2 EXPERIMENTAL

7.2.1 Effect of Temperature on the Resistance of B. stearothermophilus NCIB 8224 Spores Produced on C-Ltd Medium to Inactivation by LTSF at a Formadehyde Concentration of 16.38 mg l^{-1}.

Test pieces of B. stearothermophilus NCIB 8224 spores produced on C-Ltd medium were prepared using the method outlined in

Section 6.2.4. The apparatus was adjusted to the selected temperature and set to deliver 1 ml of 38% w/v formaldehyde solution (16.38 mg l^{-1}) at the start of the 'hold period'. The test pieces were introduced into the chamber after the initial steam and vacuum pulses to avoid physical loss of spores from the carriers (Section 6.2.3). Samples were then retrieved at defined time intervals and aseptically transferred to 1% w/v glycine as inactivator. The samples were subjected to ultrasonication (51 kHz) for 5 minutes (Section 6.3.1) and surviving organisms recovered on Nutrient Agar using the spread plate technique. Plates were incubated at 56°C for 5 days. The temperatures studied were 68°C, 70°C, 73°C, 75°C, 76°C and 80°C. Replicate inactivation experiments were performed for each temperature for exposure periods up to 70 minutes since prolonged exposure periods did not influence the number of surviving organisms and the data obtained at such periods were not reproducible (Section 6.3.4). The data from the replicate determinations were pooled and the best lines of fit determined to construct the mean survivor curves, displayed in Figs. 42-47. Figure 48 is a composite plot of all the mean survivor curves with the data points omitted for clarity and easy visual analysis.

The individual survivor curves show a wider scatter in the data points than was observed previously in inactivation studies with formaldehyde in aqueous solution (Chapter 5). It was suspected that conditions inside the chamber may not be highly reproducible and in any case could not be monitored accurately. This could explain the observed variations in experimental data. Bi-phasic

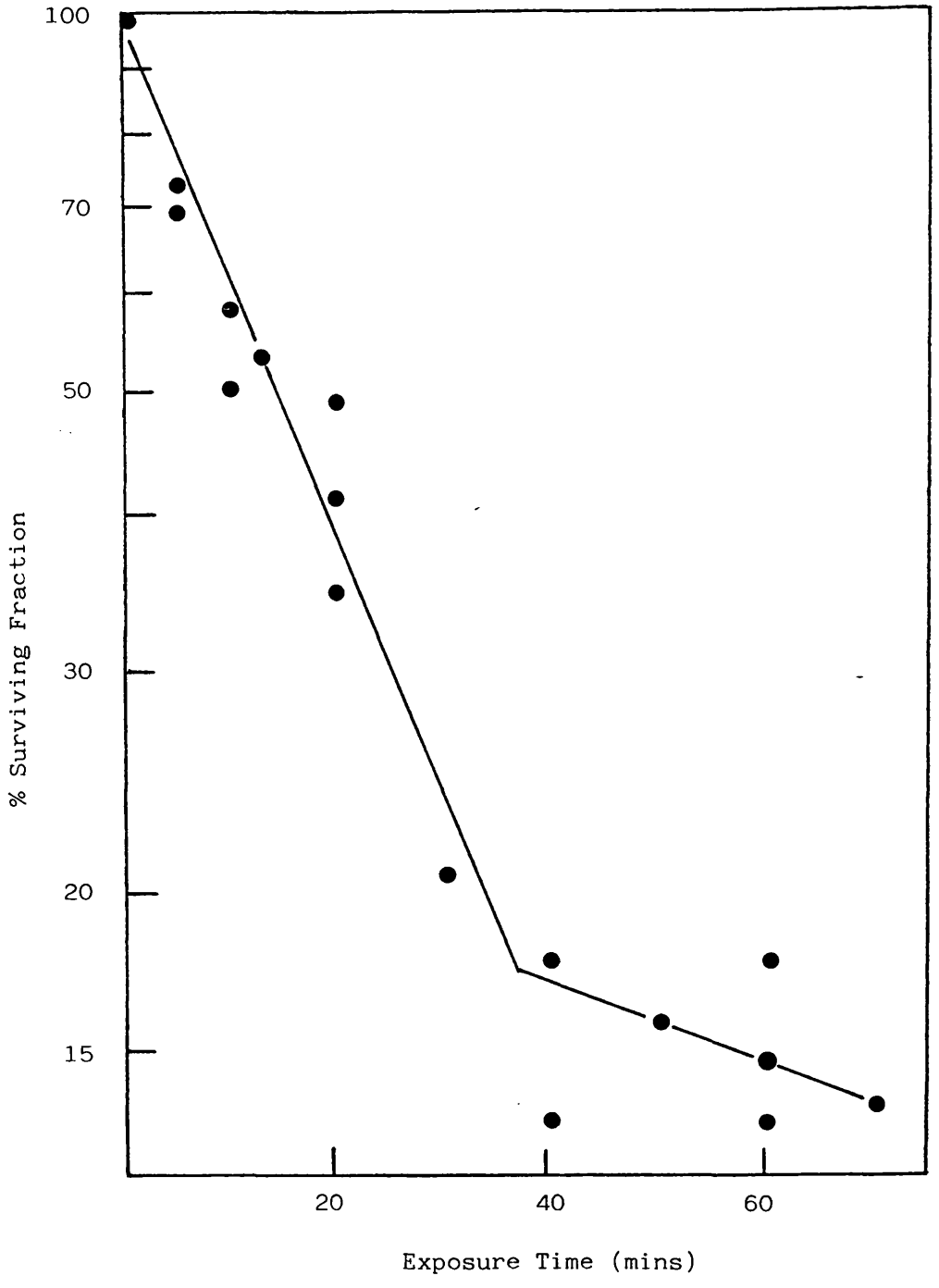


Figure 42. Inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium by LTSF at 68°C ± 0.5°C and a formaldehyde concentration of 16.38 mg l^{-1}.

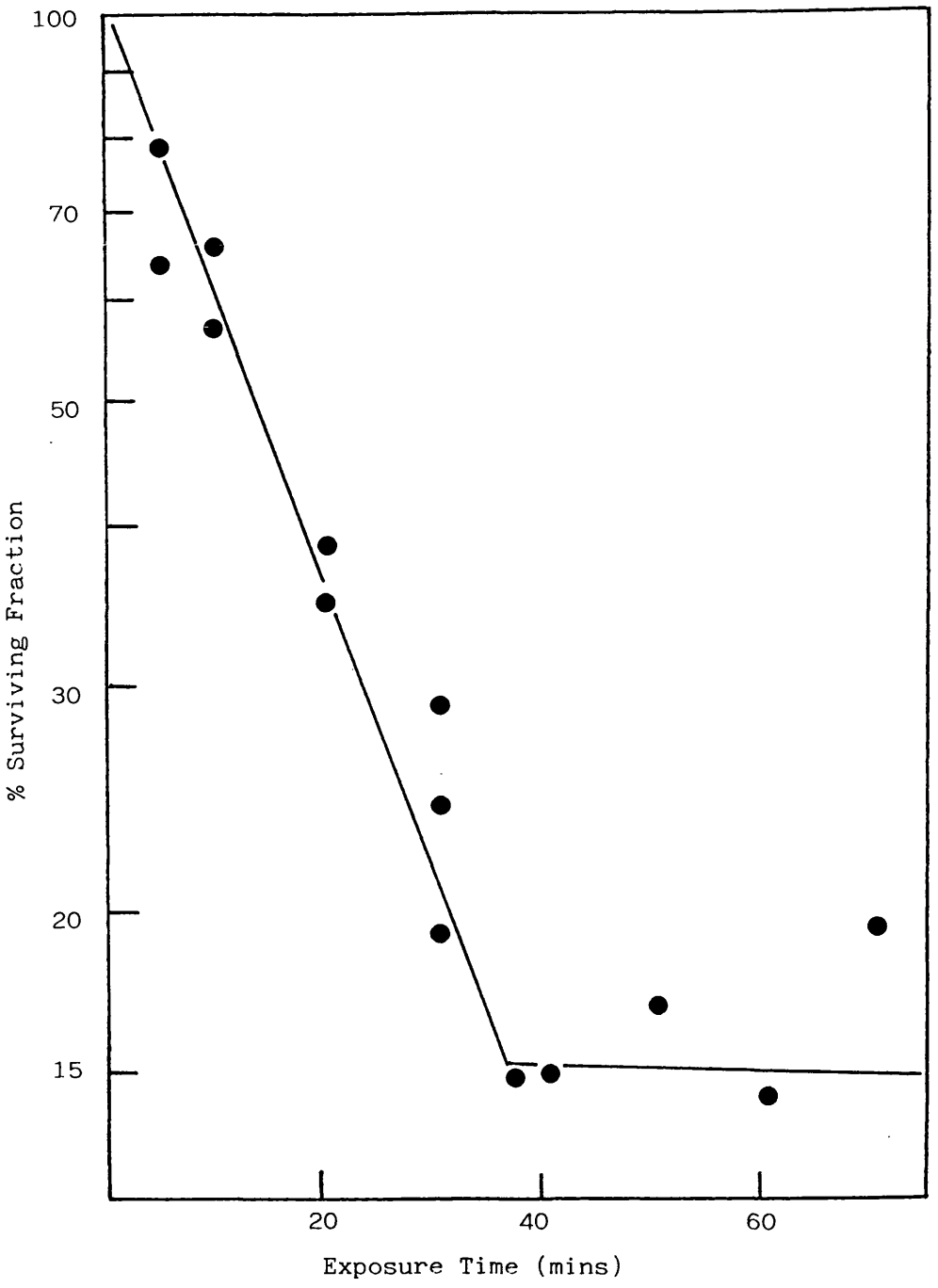


Figure 43. Inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium by LTSF at 70°C ± 0.5°C and a formaldehyde concentration of 16.38 mg l^{-1}.

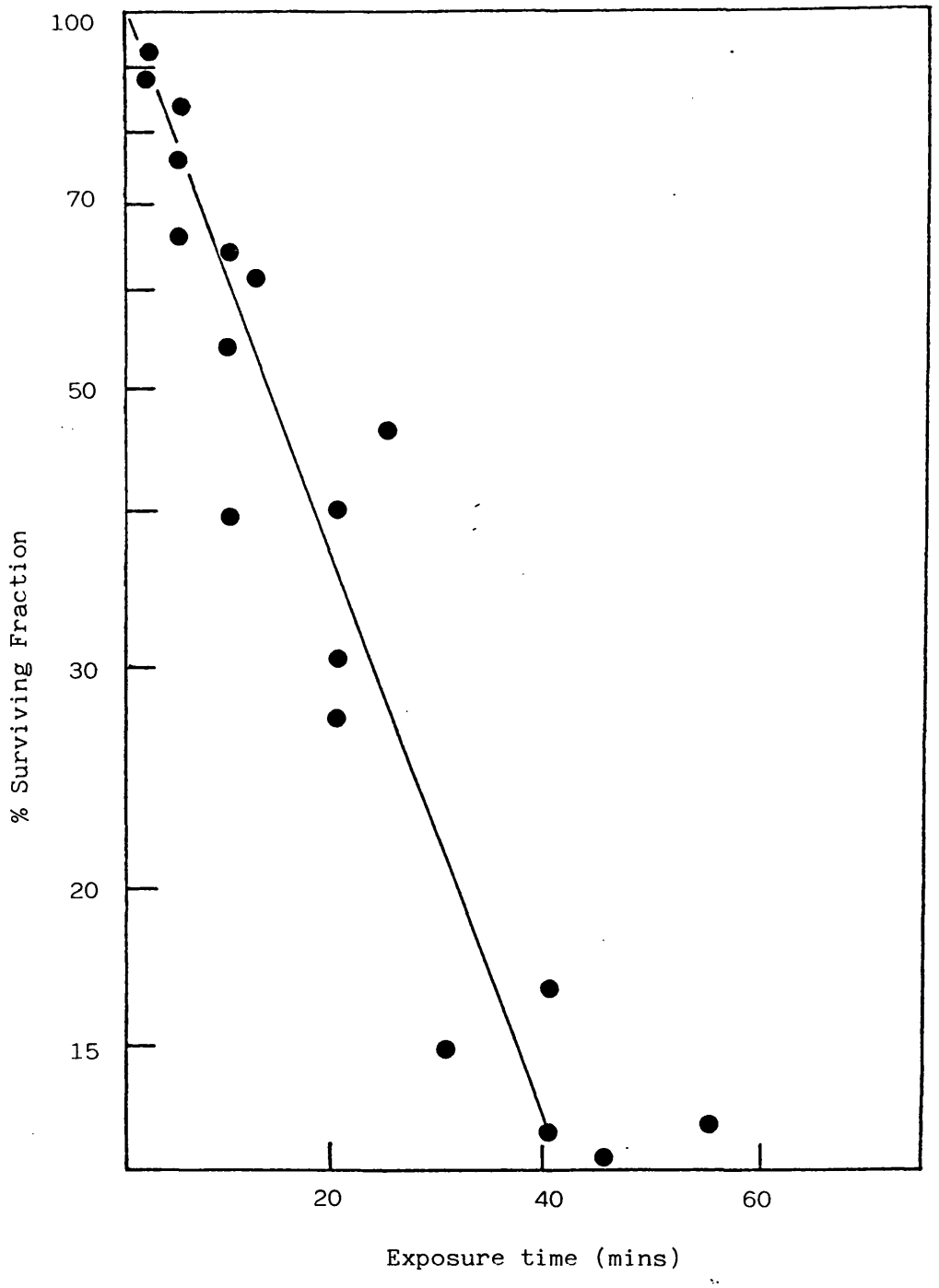


Figure 44. Inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium by LTSF at 73°C ± 0.5°C and a formaldehyde concentration of 16.38 mg l^{-1}.

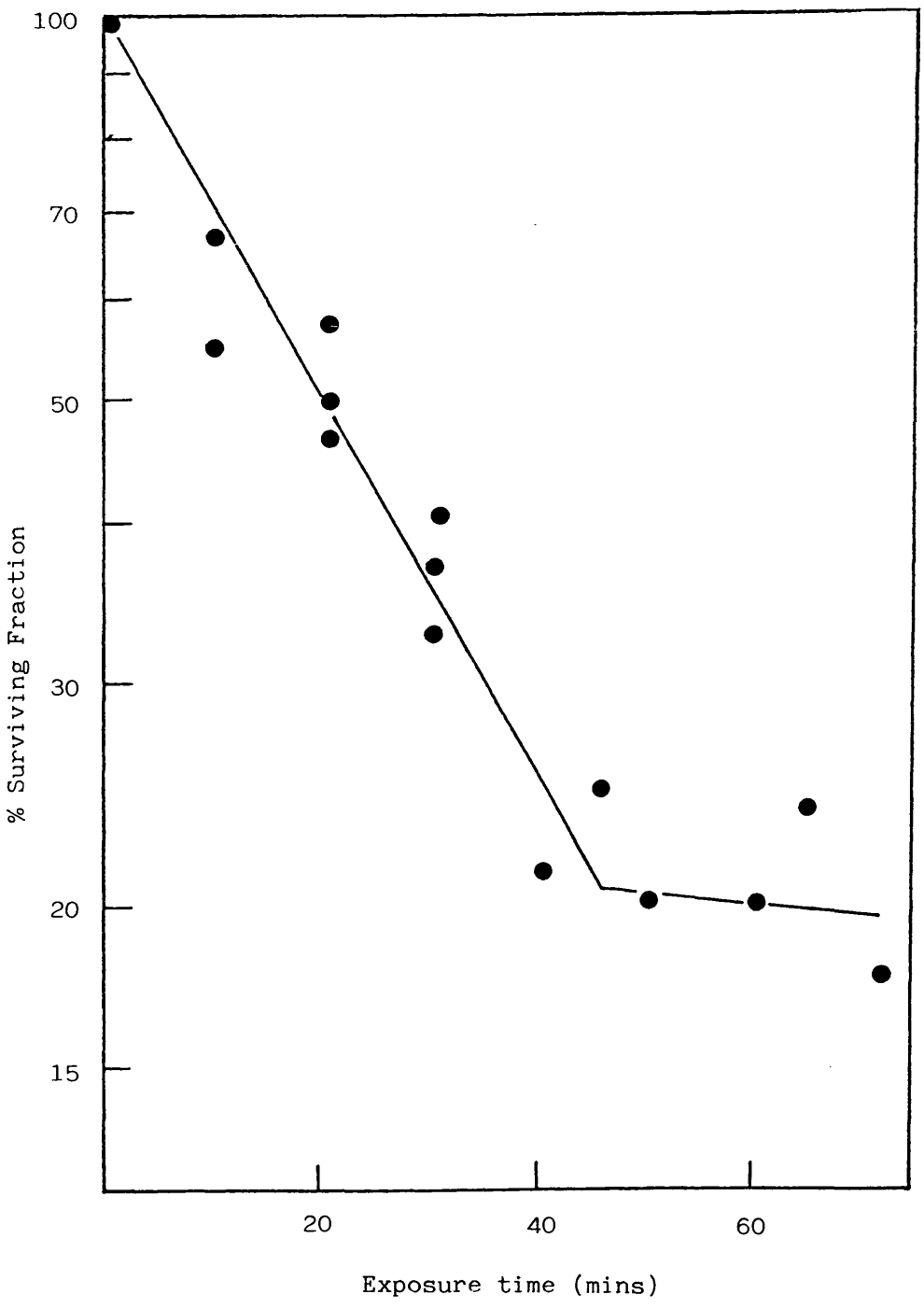


Figure 45. Inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium by LTSF at 75°C ± 0.5°C and a formaldehyde concentration of 16.38 mg l^{-1}.

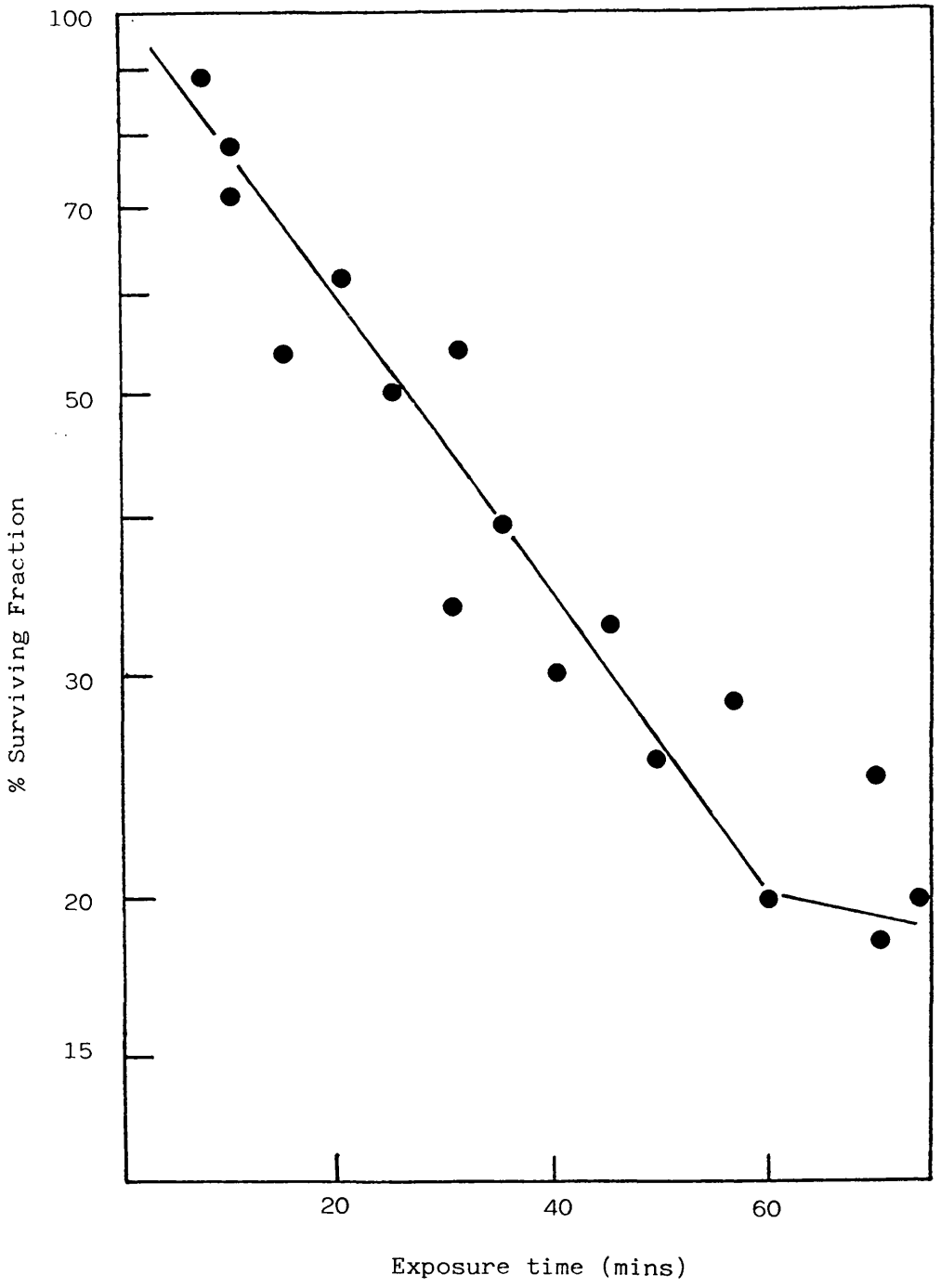


Figure 46. Inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium by LTSF at 76°C ± 0.5°C and a formaldehyde concentration of 16.38 mg l^{-1}.

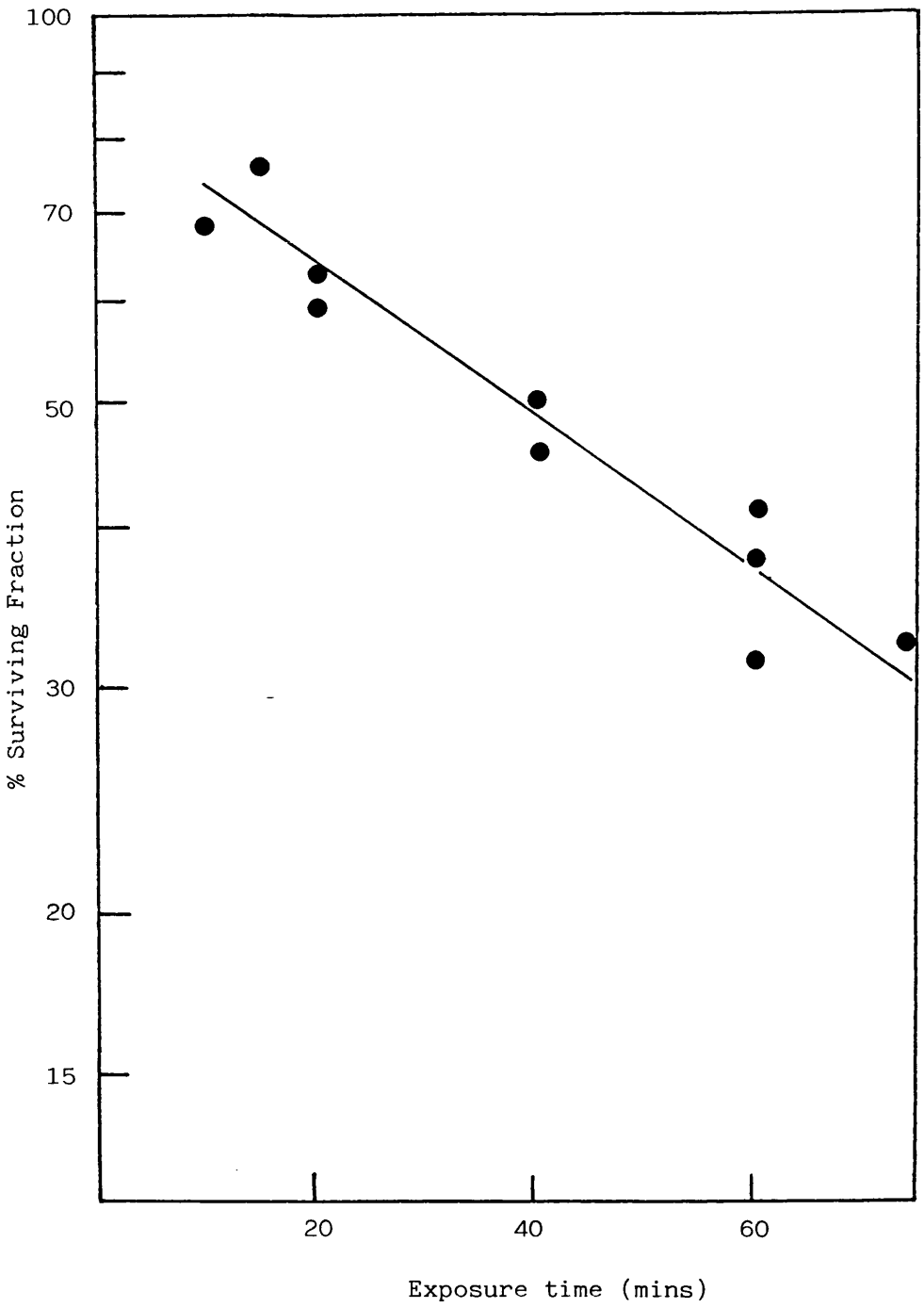


Figure 47. Inactivation of spores of B stearothermophilus NCIB 8224 produced on C-Ltd medium by LTSF at 80°C ± 0.5°C and a formaldehyde concentration of 16.38 mg l^{-1}.

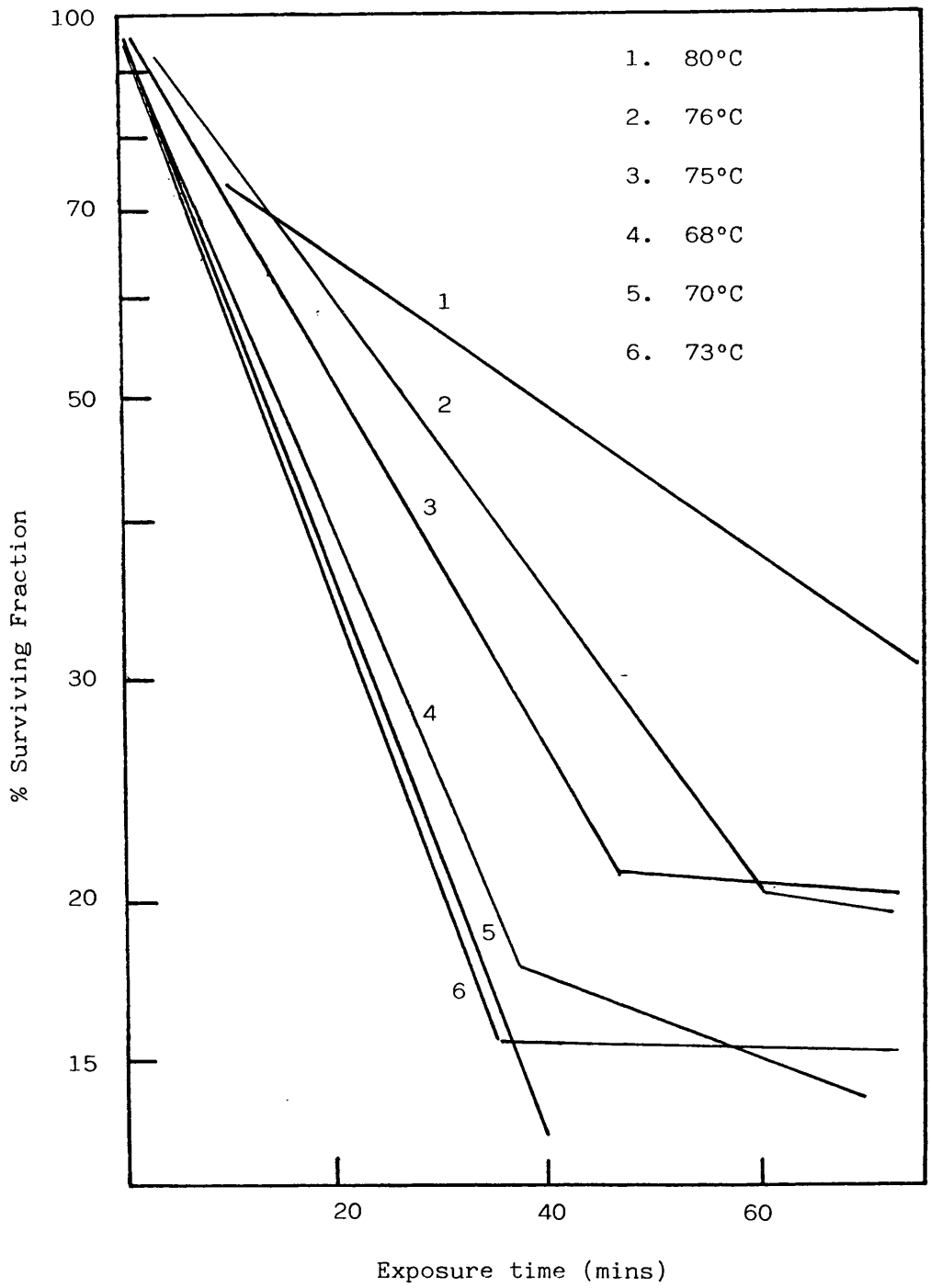


Figure 48. Composite graph showing the effect of temperature on inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd by LTSF at 16.38 mg l^{-1} using the modified Miniclave 80.

(Data points omitted for clarity).

inactivation kinetics observed during the evaluation of the modified Miniclave 80 (Section 6.3.4) were apparent in some but not all of the survivor curves displayed in Figs. 42-47.

At operational temperatures between 68° and 73°C, increase in temperature was observed to have very little effect on the lethality of the process. Over the temperature range 75° to 80°C, the results show an increased sensitivity of the spores to the test conditions as the operational temperature decreased.

It is customary for inactivation data, where temperature of inactivation is a variable, to be expressed as an Arrhenius plot which will be expected to be linear and with a negative slope. The "pseudo-Arrhenius" plot was derived to relate inactivation kinetics to the temperature of inactivation (Chapter 5) when the plot of the logarithm of the reciprocal of the t_3-value, on a log scale against the reciprocal of the Absolute temperature, was considered more appropriate for cases where the survivor curve shapes deviated from the log-linear pattern. Then, $3/t_3$ was considered a more accurate estimation of the inactivation rate constant 'k' than 1/D. Since survivor curves obtained in this investigation were bi-phasic and linear for much less than one log cycle (Figs. 42-47) it was not practical to read t_3-values from these survivor curves. However, the problem was overcome by projecting the initial log-linear portions of the bi-phasic survivor curves to the exposure time axis where the projected D-values were read in minutes. Figure 49 shows the plot of the reciprocal of the projected D-value, on a log scale

as a function of the reciprocal of the corresponding Absolute temperature, for the data displayed in Figs. 42-47. There was no observed linearity in the data points displayed in Fig. 49. Unlike the Arrhenius plot when the plot would be expected to be linear and with a negative slope, the data displayed in Fig. 49 revealed evidence of a positive slope.

7.2.2 Effect of Formaldehyde Concentration on the Resistance of *B. stearothermophilus* NCIB 8224 Spores Produced on C-Ltd Medium to Inactivation by LTSF AT 76°C

Test pieces of spores of *B. stearothermophilus* NCIB 8224 produced on C-Ltd medium were exposed to LTSF treatment in the modified Miniclave 80 using the procedure outlined in Section 6.3.4. The machine was adjusted to maintain an operational temperature of 76°C ± 0.5°C throughout the investigation. The volume of 38% w/v formaldehyde (containing 10% v/v methanol as stabilizer) injected into the vaporiser at the beginning of the hold period and subsequently as gaseous formaldehyde, into the chamber was either 0.5 ml, 1 ml, 2 ml or 3 ml giving formaldehyde concentrations in the chamber of 8.19 mg l^{-1}, 16.38 mg l^{-1}, 32.76 mg l^{-1} or 49.14 mg l^{-1} respectively. For each formaldehyde concentration, samples were withdrawn at defined time intervals and the number of surviving organisms determined as described previously (Section 6.3.1). Replicate determinations were made at each formaldehyde concentration and mean survivor curves were constructed from the data.

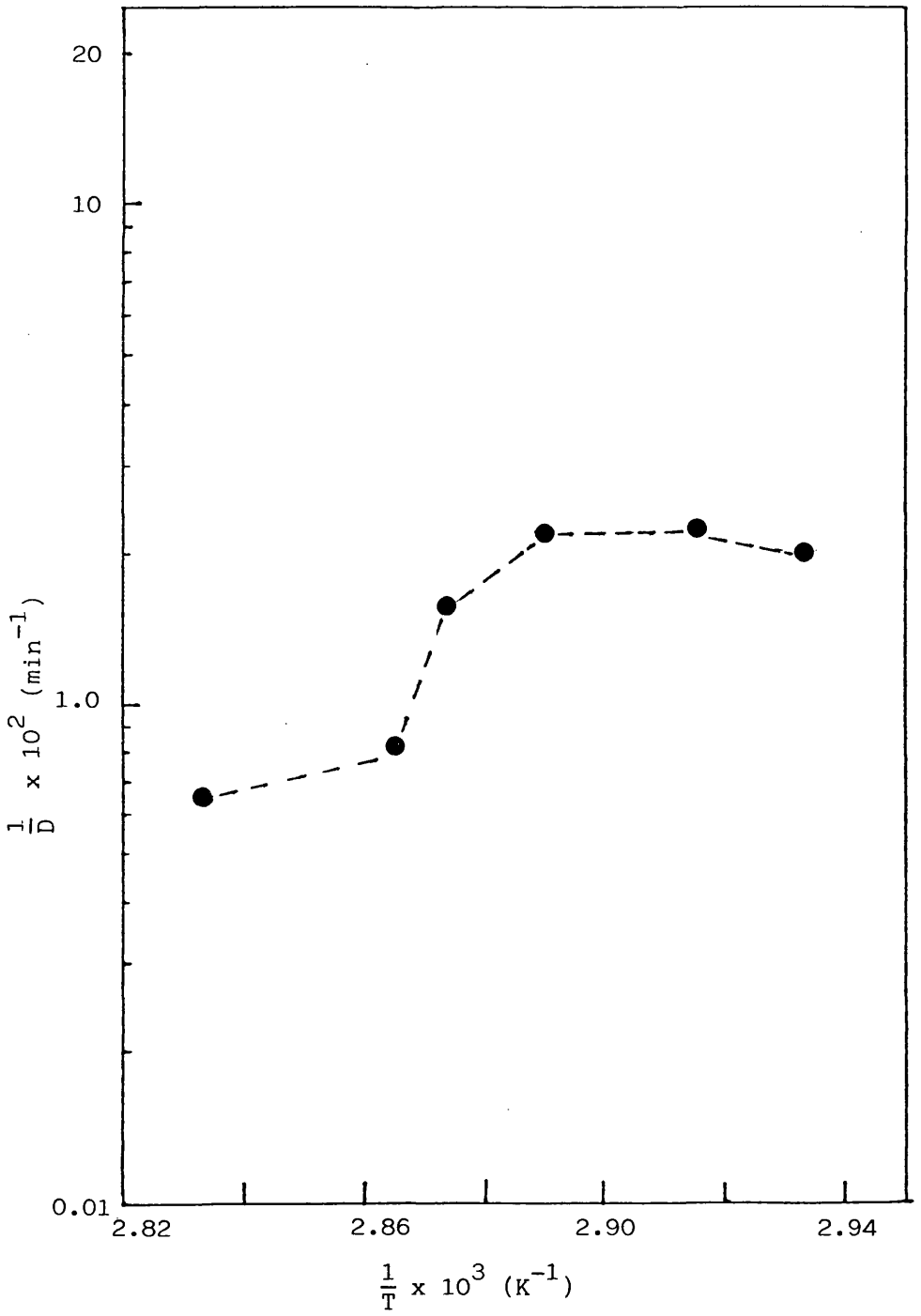


Figure 49. Arrhenius plot of the reciprocal of the projected D-value, on a log scale, against the reciprocal of the Absolute temperature for the inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium by LTSF at 16.38 mg l^{-1} formaldehyde concentration.

Data displayed in Fig. 50 show the effect of formaldehyde concentration on the inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium by LTSF at 76° ± 0.5°C. An increase in sensitivity to the test conditions was observed as the formaldehyde concentration was increased from 8.19 mg l^{-1} to 49.14 mg l^{-1}. These formaldehyde concentrations were only theoretical as there was no facility at the time of this investigation to accurately measure formaldehyde concentrations inside the chamber. However, as expected, the results indicated a significant effect of formaldehyde concentration on resistance of bacterial spores exposed to LTSF conditions. The data displayed in Fig. 50 were summarised by plotting the projected D-value on a log scale against the corresponding chamber formaldehyde concentration on a linear scale (Fig. 51).

7.2.3 Measurement of Formaldehyde Concentrations in the Chamber of the Modified Miniclave 80

The primary requirement of a sterilizer is that it should maintain the conditions for sterilization in all parts of the chamber for the required time. While the temperature and pressure in the Miniclave 80 can be monitored accurately during its operation, it is not possible to monitor the formaldehyde concentration. Indeed, this is one of the reasons that biological indicators are necessary in LTSF sterilization. The reduced reproducibility observed with inactivation data obtained using the Miniclave 80 (Sections 6.3.4 and 7.2.1) and the bi-phasic nature of

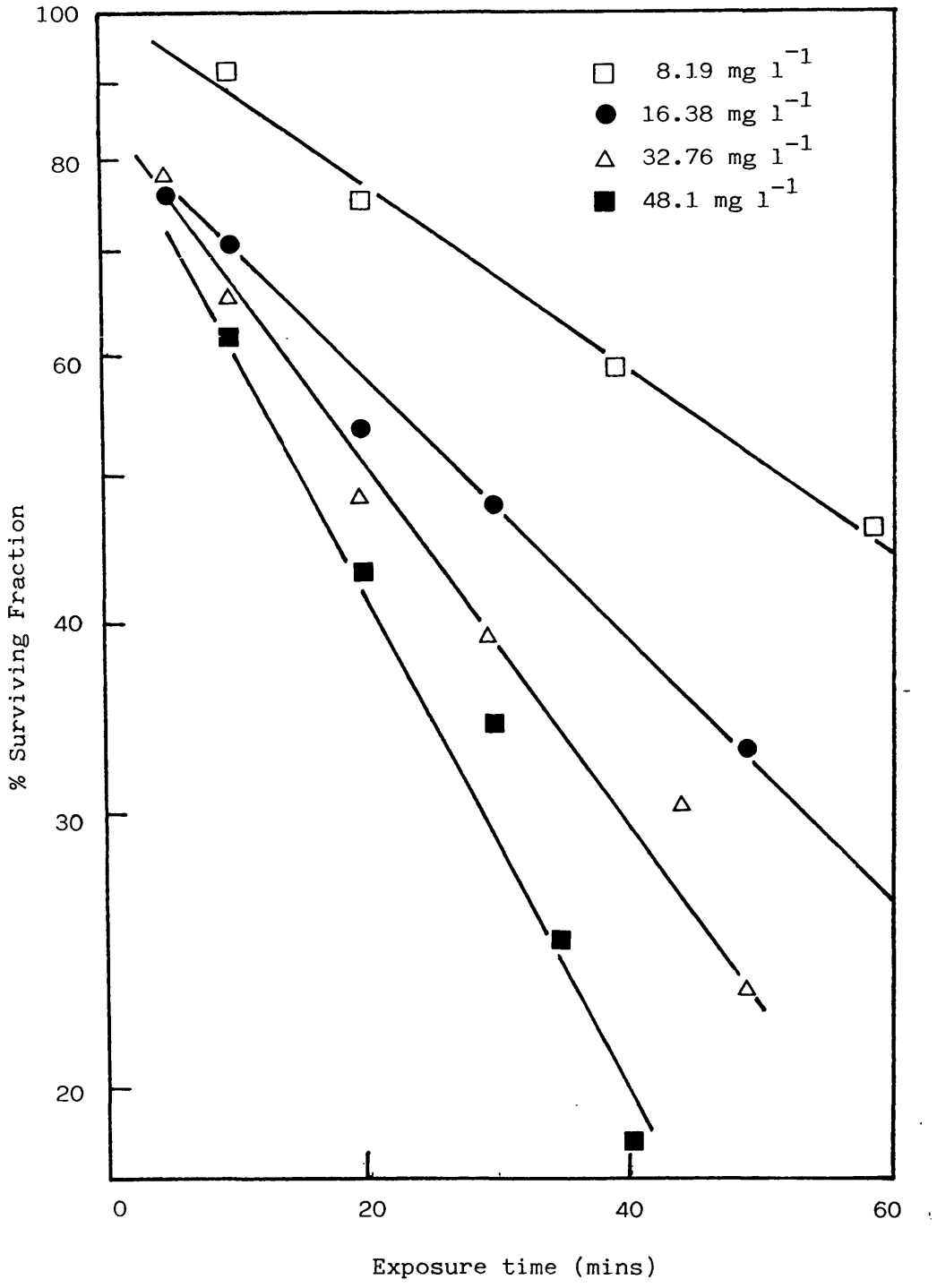


Figure 50. Effect of formaldehyde concentration on the inactivation of spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium by LTSF at 76°C ± 0.5°C using the modified Miniclave 80.

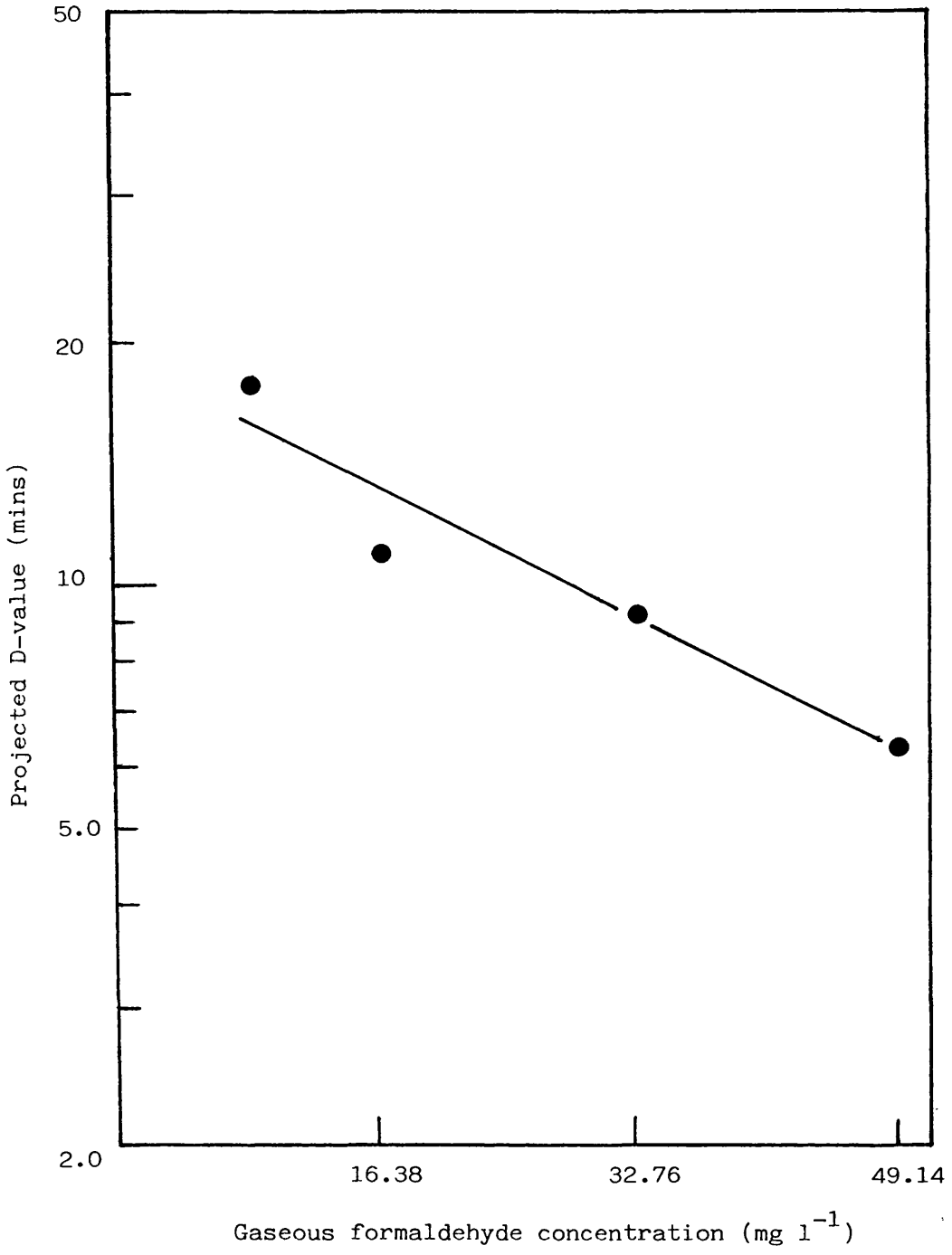


Figure 51. Plot of the projected D-value on a log scale against the corresponding concentration of gaseous formaldehyde for the inactivation of spores of B. stearothermophilus NCIB 8224 by LTSF at 76°C ± 0.5°C.

the survivor curves could be due to variations in formaldehyde concentration in the chamber during the exposure period. While it is not possible to measure the concentration of formaldehyde in the chamber of the modified Minicalve 80 during operation, it could be indirectly determined by assaying samples from the effluent vessel for dissolved formaldehyde using the sodium sulphite titration method (109).

50 ml of molar sodium sulphite solution (BDH Ltd) was transferred to a 100 ml Erlenmeyer flask followed by the addition of three drops of thymolphthalein indicator solution (0.1% w/v in ethanol). The contents were neutralised with 2-3 drops of normal hydrochloric acid. An accurately measured volume of the formaldehyde solutions obtained from the effluent vessel was then added until a blue colouration developed. The resulting mixture was then titrated slowly with normal hydrochloric acid until discolouration. 1 ml of normal hydrochloric acid would be equivalent to 0.03003 g of formaldehyde. The amount of dissolved formaldehyde in the aliquot and in the volume contained in the effluent vessel was then determined. The cumulative amount of dissolved formaldehyde was then plotted as a function of hold time. These determinations were carried out at operational temperatures of 68°C, 73°C, 76°C and 80°C. The machine was adjusted to deliver 2 ml of 38% w/v formaldehyde solution, equivalent to 32.76 mg l^{-1} gaseous formaldehyde in the chamber. This concentration is also equivalent to 0.76 g formaldehyde in the chamber assuming all the injected formaldehyde solution was fully vapourised and admitted to

the chamber, without any amount being evacuated from the chamber (Fig. 52).

Data displayed in Fig. 52 show the variation between the amount of formaldehyde detected in the effluent vessel with hold time at different operational temperatures. These results show that the amount of dissolved formaldehyde collecting in the effluent vessel increased to a maximum value after approximately 15 minutes of hold time. This implies rapid elution of gaseous formaldehyde from the chamber during the initial 15 minutes of hold period. Furthermore, the amount of available formaldehyde, i.e. the difference between the profile and theoretical chamber concentration assuming no evacuation after injection of 2 ml of 38% w/v formaldehyde solution, decreases with increasing operation temperature. To illustrate this observation more clearly, the amount of available formaldehyde after 30 minutes of hold period for different temperatures, was plotted on a linear scale as a function of the corresponding operational temperature (Fig. 53).

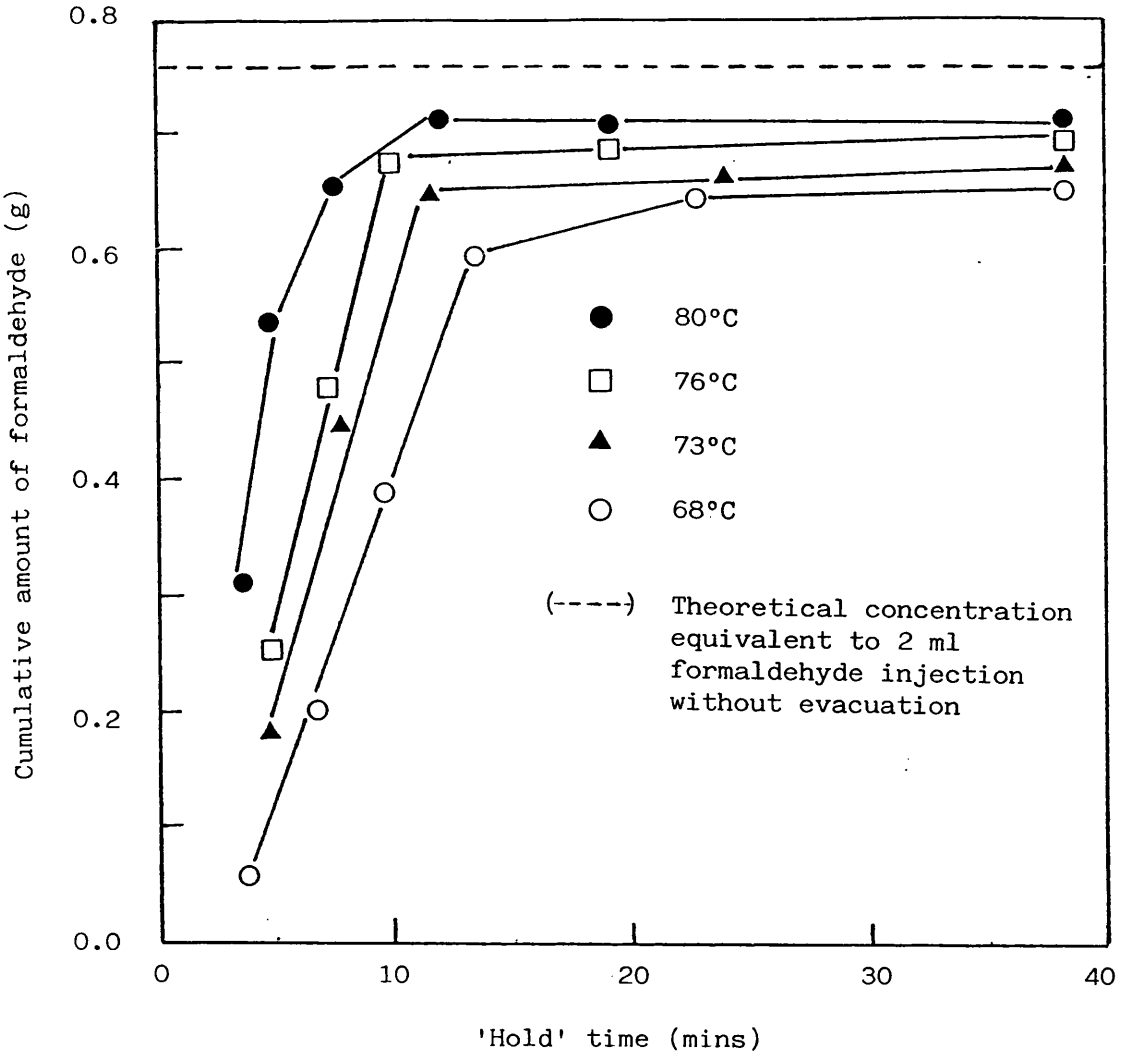


Figure 52. The effect of temperature on the variation of the amount of dissolved formaldehyde collecting in the effluent vessel during the hold period following the injection of 2 ml of 38% w/v formaldehyde solution to the vaporizer of the modified Miniclave 80.

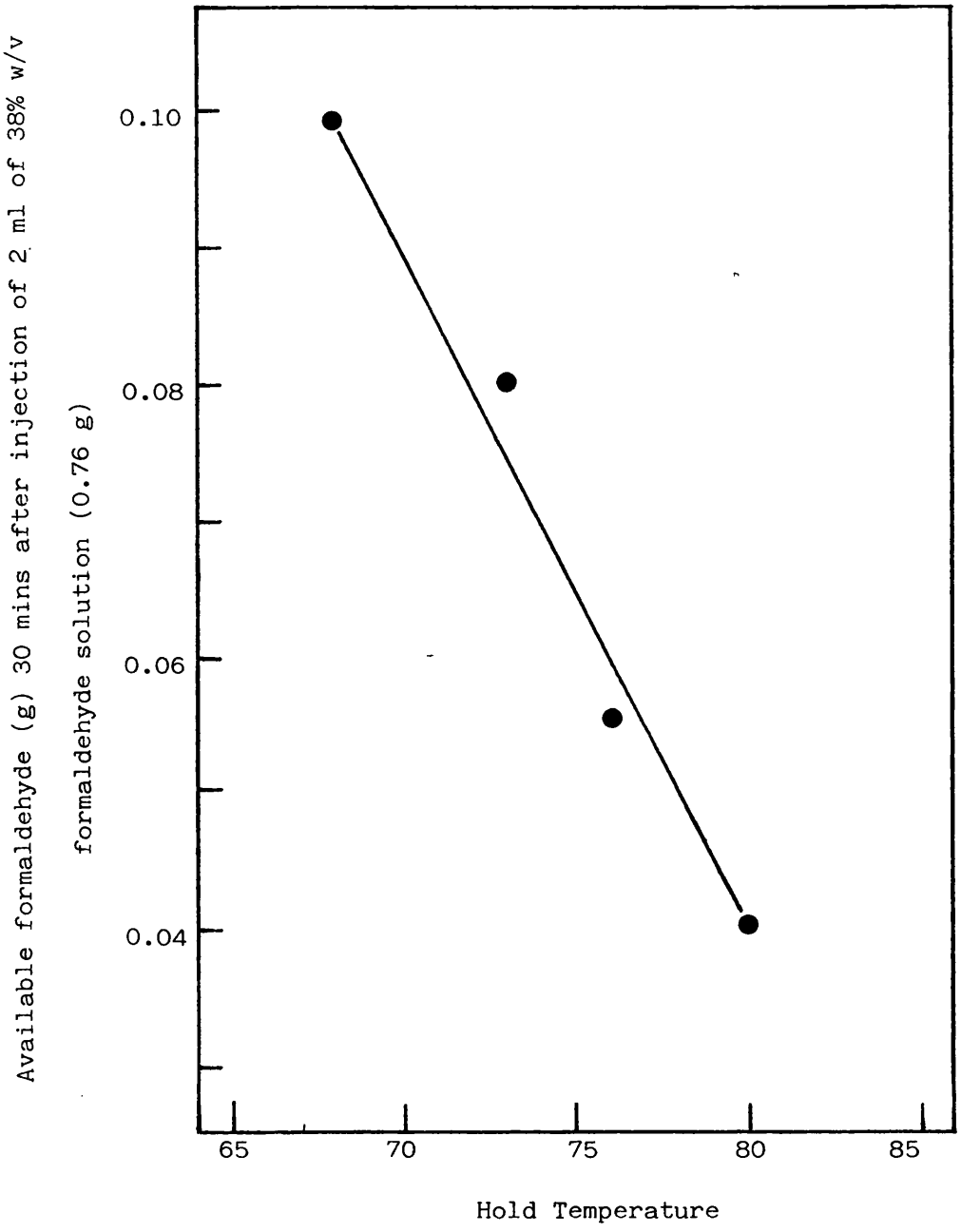


Figure 53. Graph to show amount of formaldehyde remaining in the chamber of the modified Miniclave 80 30 mins after injection of 2 ml of 38% w/v formaldehyde solution at various operational temperatures.

7.3 DISCUSSION

Experiments described in this chapter were carried out as a preliminary investigation of the effects of temperature and formaldehyde concentration on the inactivation of spores of B. stearothermophilus NCIB 8224, produced on C-Ltd medium, by LTSF using the modified Miniclave 80. The ranges of operational temperature (68°C - 80°C) and formaldehyde concentration (8.19 - 49.14 mg l^{-1}) considered in this investigation covered the ranges that have been used in commercial LTSF sterilizers (2). Spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium were used in the investigations described in this chapter since they were selected from characterisation studies that have been reported in this thesis, as the best candidate for development into a biological indicator for LTSF processes.

Few reports based on a growth or no growth criterion have been published describing the sporicidal properties of the LTSF process using commercial apparatus. However, there is little information that has been published to determine, quantitatively, the resistance of bacterial spores to various conditions of LTSF. Problems assocted with the recovery of samples from LTSF environments and the subsequent determination of the number of surviving organisms has hampered the construction of survivor curves to describe bacterial spore response to LTSF treatment.

Pickerill (232) demonstrated the sporicidal activity of LTSF

when no surviving organisms could be recovered after exposing spore strips of B. stearothermophilus, B. pumilus, B. globigii and Cl. sporogenes to LTSF treatments. The cycles consisted of an initial formalizing stage followed by a 'hold period' of 20 minutes with a formaldehyde concentration of about 50 mg l^{-1} at temperatures between 65° and 80°C. Alder (126) reported no survivors after exposing 4.8 x 10^5 spores of B. subtilis and 9.0 x 10^5 spores of B. stearothermophilus to 100 mg l^{-1} gaseous formaldehyde at temperatures between 80°C and 90°C for 35 and 40 minutes respectively. Hurrell et al. (137) described a method of isolating samples from the chamber of a steam formaldehyde sterilizer in an attempt to describe the inactivation process by a survivor curve but encountered problems of recovering low numbers of survivors and the problems of physical loss of spores from carriers caused by the vacuum and steam pulses. Hoxey (22) designed an experimental LTSF apparatus based on a manifold system and demonstrated substantial reductions in the viable count in short periods of time, of all the spores exposed to LTSF at 80°C and 27 mg l^{-1} formaldehyde concentration.

As part of an ongoing research project on LTSF sterilization, the experiments described in this chapter can only be considered preliminary in evaluating the performance of the modified Miniclave 80. The results obtained revealed reduced reproducibility in the inactivation data and bi-phasic inactivation kinetics were apparent in some of the survivor curves. Variations in the concentration of formaldehyde in the chamber were suspected

to cause the reduced reproducibility. There have been reports of decaying formaldehyde concentrations in steam-formaldehyde sterilizers during the LTSF cycle (131, 137). Marcos and Wiseman (131) used a Modified Miniclave 80 to demonstrate that the concentration of formaldehyde in the chamber atmosphere was not homogeneous and that it decreased rapidly with time. It was suspected that a decrease or disappearance of formaldehyde available for inactivation, could be the cause of the bi-phasic nature of the survivor curves obtained. The investigation into the fate of the administered formaldehyde (Section 7.2.3) showed that most of the formaldehyde was eluted from the chamber during the initial 20 minutes of the hold period (Fig. 52). Furthermore, formaldehyde was shown to be eluted from the chamber at rates which increased with increasing operational temperature (Fig. 52). The amount of formaldehyde available for inactivation of the bacterial spores decreased as the temperature was increased (Fig. 53). It was assumed in this investigation that all the formaldehyde injected into the vaporizer was fully vaporized and entered the chamber. It is quite possible that some of the formaldehyde vapour went into solution before entering the chamber and some remained within the pipework of the apparatus. The method used to demonstrate the formaldehyde decay (Section 7.2.3) may not give an absolute measure of the formaldehyde concentration at any given time neither could it be expected to detect various forms of formaldehyde species in the chamber. The method was however considered sufficient to show that there was a definite progressive loss of formaldehyde from the chamber atmosphere. The modification carried out on the Miniclave

80 (Section 6.2.1) allowed the volume of administered formaldehyde to be adjusted to a defined amount. However, the temperature control used, maintained the set temperature by continually admitting steam and evacuating it at rates that increased with increasing temperature. It is possible that this feature in the design caused the formaldehyde levels to decay in the described fashion.

Further modifications are underway to enable multiple injections of appropriate formaldehyde aliquots at defined time intervals to maintain the constant sterilant levels during the hold period (159). An accurate and direct method to determine concentrations of formaldehyde in the chamber during the LTSF cycle is obviously required to determine the amounts of formaldehyde solution to be injected and the frequency of formaldehyde injection to the vaporizer. In practice, some commercial cycles use formaldehyde vapour and steam during the initial steam pulse stage or use formaldehyde vapour on its own to evacuate a dry chamber (133). Alder (133) reported that the sporicidal effect of formaldehyde with low temperature steam sterilization (FLTS) occurred during the formalizing stage and that the hold period was mainly concerned with the removal of residual formaldehyde from the chamber.

Despite the limitations of the current modification on the Miniclave 80, the results reported in this chapter confirm the sporicidal activity of LTSF and also show that temperature and

formaldehyde concentration have a significant effect on the efficiency of the LTSF processes.

Temperature variations between 68°C and 73°C did not have a significant effect on the lethality of the process. When spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium were exposed to LTSF at a formaldehyde concentration of 16.38 mg l^{-1}, approximately 80% of the spores were inactivated over 45 minutes at 68°C (Fig. 42) and approximately 85% were inactivated over 45 minutes at 73°C (Fig. 44). However, the test spores showed increased resistance as the temperature was increased from 75°C to 80°C. Approximately 80% of the spores were inactivated over 45 minutes at 75°C (Fig. 45) and approximately 30% were inactivated over 65 minutes at 80°C (Fig. 47). The decrease in lethality observed with increasing operational temperature on the LTSF cycle of the modified Miniclave 80, is in accordance with the observed decline in the amount of formaldehyde available for the inactivation of the spores (Section 7.2.3). Data displayed in Fig. 53 show that after 30 minutes of exposure at hold conditions, approximately 5% of the originally administered formaldehyde was available for inactivation at 80°C compared to about 13% at 68°C. The U.K. recommended temperature for LTSF sterilization is 73° ± 2°C. There is however need for lower temperatures of around 55°C for the sterilization of even more temperature sensitive medical equipment like electronic and optical surgical instruments (133, 141).

It is important to note that various temperatures investigated to inactivate spores of B. stearothermophilus NCIB 8224, by LTSF require different steam pressures. These pressures vary between 0.475 bar absolute at 80°C to 0.25 bar absolute at 65°C (134). Equal amounts of formaldehyde were used and were expected to produce equal partial pressures within the chamber at each temperature and pressure combination. However due to variation in chamber pressure at different temperatures, the mole fraction of formaldehyde was expected to increase as the temperature decreased.

When the reciprocal of the projected D-values for the inactivation of the test spores by LTSF at 16.38 mgl^{-1} at temperatures between 68°C and 80°C was plotted against the reciprocal of the Absolute temperature, the results did not reveal any linearity expected of an Arrhenius relationship (Fig. 49). It is possible that the data points displayed in Fig. 49 were part of a linear relationship, since these data were derived from less than 1 log cycle of the survivor curves displayed in Figs. 42-47. There is however clear evidence of decreased lethality with increasing temperature.

LTSF is a multiparameter process and therefore sporicidal activity cannot be related to any of the parameters in isolation. When the effect of formaldehyde concentration on the inactivation of the test spores by LTSF at 76°C was investigated, an increase in the sensitivity of the spores was observed as the concentration increased from 8.19 mg l^{-1} to 49.14 mg l^{-1} (Fig. 50). Several LTSF

cycles have been reported to use formaldehyde concentrations between 3.3 mg l^{-1} and 100 mg l^{-1} at temperatures between 60°C and 80°C (2). Although increasing formaldehyde concentration tends to increase the lethality of the LTSF process, an upper limit has to be defined to reduce the risks of operator safety, to minimise toxic residuals on sterilized loads and to avoid mechanical failures due to polymer depositions.

The effects of temperature and formaldehyde concentration on the resistance of spores of B. stearothermophilus NCIB 8224 have been investigated separately. The exact roles of heat from low temperature steam and that of the formaldehyde monomer in the combined sporicidal action are still not clearly understood. From the results obtained in the investigations reported in this chapter, variation in formaldehyde concentration produced greater differences in the response of the test spores than variation in temperature, especially at lower temperatures between 68°C and 73°C. It is possible that latent heat from low temperature steam could be providing enough energy to produce changes in the spore structure after which the lethality of formaldehyde is increased. The energy from low temperature steam could cause an increase in the permeability of formaldehyde to the reactive sites in the protoplast. Intact spore coats have been reported to offer no protection from glutaraldehyde to spores of B. pumulis (58). Dadd and Daley (53) reported that increased permeability in coat-defective and chemically treated spores did not increase their sensitivity to ethylene oxide even though resistance to lysozyme

was reduced. It is therefore difficult to imagine the spore coats being impermeable to molecules of the size of formaldehyde. The passage of ethylene oxide to the protoplast could be hindered by non-lethal alkylation of thiol groups in the spore (53) and this could be applied to the case of formaldehyde. Until the exact mechanism of LTSF induced lethality in bacterial spores is established, the roles of different parameters of LTSF sterilization cannot be easily explained but that a synergism between all the parameters involved in the LTSF process is necessary for the sporicidal activity.

CHAPTER 8

CONCLUDING DISCUSSION

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Low Temperature Steam and Formaldehyde (LTSF) sterilization has become widely accepted in N.W. Europe and the UK as suitable for the sterilization of thermolabile medical equipment. There is however little information published on the practical use of LTSF. LTSF has many advantages over ethylene oxide sterilization (EO) but there is uncertainty in the reliability as sterilizers, of the machines and cycles currently avaialble. There are also differences of opinion on the relative roles of the parameters involved in the process.

With a process such as LTSF in which there is potential in variation in the many parameters involved, only biological indicators can integrate all these variables to successfully monitor the process. Biological indicators have traditionally been prepared from bacterial spores exhibiting high resistance to the sterilizing conditions. The spores selected for development as biological indicators for LTSF sterilization should possess a high germination index and a high resistance to each parameter in isolation to confirm the achievement of a sporicidal combination. The bacterial strain chosen should be capable of producing high spore yields from chemically defined media, should be aerobic and non-pathogenic. As yet, there is no internationally standardized biological indicator for LTSF sterilization and this could be

hampering its practical application in the health services.

The work described in this thesis is part of an ongoing research project on LTS disinfection and LTSF sterilization undertaken to characterize spores of selected Bacillus strains in terms of their growth and sporulation, ease of harvesting and cleaning, germination index and resistance of the spores to constituent parameters of LTSF. Thus the resistance of the spores to inactivation by moist heat was first determined followed by determination of the resistance to inactivation by formaldehyde, looking initially to inactivation by formaldehyde in aqueous solution. The effect of the composition of chemically defined media on the above characterization was examined.

The effect of temperature on the resistance of the spores of the selected Bacillus strains to inactivation by 0.5% w/v aqueous formaldehyde was investigated at temperature regions that have been reported for use in various LTSF protocols. Since spores to be used as biological indicators may be stored in aqueous suspension for considerable periods of time, the stability of the spores exhibiting desirable characteristic was investigated with respect to their resistance to inactivation by formaldehyde in aqueous solution when stored at 4°C.

Finally, a modified commercial LTSF apparatus was used to investigate the resistance of the best contender for development as a biological indicator, to inactivation at LTSF conditions. The

effects of temperature and formaldehyde concentration to the inactivation of the spores by LTSF were investigated. Initial steam and vacuum pulses were observed to physically remove spores from carrier surfaces. The problem was overcome by introducing the test pieces to the sterilizing conditions after the initial steam and vacuum pulse stage. The modified apparatus had further drawbacks in that the chamber formaldehyde levels were demonstrated to decay rapidly with time at rates accelerated by increasing the hold temperature. Experiments using the modified apparatus were therefore restricted to the first 60-70 minutes of the 'hold' period.

Experiments described in Chapter 3 were conducted to examine the growth and sporulation, incubation time necessary for sporulation, ease of harvesting and cleaning and to determine spore yields and germination indices of the selected Bacillus strains. The composition of the chemically defined sporulation medium was observed to influence these characteristics in the tested strains. Solid CDM was observed to perform better than liquid media. Of the two compositions of CDM considered, C-Ltd medium produced higher spore yields in as little as 2 days. Furthermore, C-Ltd medium was simpler to prepare and spores harvested from it were easier to clean. Based on the criterion of the above mentioned characteristics, B. stearothermophilus NCIB 8224 and NCTC 10003 were shortlisted for further investigation. Bacillus stearothermophilus NCIB 8224 produced a high spore yield (95%) in 2 days when grown on C-Ltd medium and the spores exhibited a germination

index of 68%. B. stearothermophilus NCTC 10003 produced a 90% spore yield in 2 days when grown on C-Ltd medium and the spores exhibited a germination index of 78%.

Experiments described in Chapter 4 were carried out to determine and compare the resistance of the spores of the Bacillus strains to inactivation by moist heat at 110°C. Chemically defined sporulation media was shown to be capable of producing bacterial spores of reproducible moist heat resistance. The composition of the sporulation medium was demonstrated to influence not only the resistance of the spores to the inactivation treatment but also the shape of the survivor curves produced. Of the thermophilic group of strains investigated, C-Ltd medium produced spores exhibiting higher resistance to moist heat at 110°C. The use of t_3-values was introduced here as a practical alternative to compare the resistance of bacterial spores when the survivor curves are not necessarily log-linear over the entire exposure periods. It was interesting to observe that spores of B. stearothermophilus NCTC 10003, produced on C-Ltd medium, and currently being considered by the LTSF Reference Laboratory (Luton College) as suitable for development as a biological indicator for LTSF, were the most sensitive of the thermophilic strains, at these test conditions (t_3 = 9.53 minutes). B. stearothermophilus NCIB 8224 spores produced on C-Ltd medium were the second most resistant (t_3 = 40 mins) with spores of B. stearothermophilus NCIB 10814 the most resistant (t_3 = 120 mins). However spores of B. stearothermophilus NCIB 10814 produced on C-Ltd medium exhibited a type D survivor curve with

evidence of activation. These spores also exhibited a low germination index, 3%, and were therefore not considered suitable for development as biological indicators for LTSF processes.

Experiments reported in Chapter 5 were conducted to compare the resistance of the spores to inactivation by 0.5% w/v aqueous formaldehyde solution. The comparison was carried out at 70°C, a convenient temperature near the official recommended range for LTSF sterilization in the UK (i.e. 73 ± 2°C). A variety of survivor curve shapes and a range of resistances was displayed, a further indication of the influence of the composition of sporulation medium. Spores of B. stearothermophilus NCIB 8224 produced on C-Ltd medium were the most resistant (t_3 = 90 minutes) with spores of B. stearothermophilus NCTC 10003 produced on C-Ltd medium, the most sensitive of the thermophilic group (t_3 = 20 mins). In the light of the observed influence of sporulation medium on bacterial spore characteristics, and the diversity observed within the stearothermophilus species, it was considered necessary to specify sporulation conditions and the strain when reporting on bacterial spore characteristics. B. stearothermophilus can wrongfully be regarded as one super resistant species forgetting the vast number of strains in this group that produce spores possessing a whole spectrum of characteristics depending on the sporulation conditions.

The data presented on the resitance of the selected spores to inactivation by 0.5% w/v aqueous formaldehyde solution at

temperatures between 60° and 80°C revealed some irregularities when the data were expressed in an Arrhenius-type relationship. Discontinuities and inflexions were apparent on the "pseudo-Arrhenius" plots at temperature regions corresponding to 70°-75°C. These irregularities could suggest different mechanisms of inactivation at temperature ranges on either side of the observed irregularities. It was also considered possible that conformational changes in the target molecule, the nucleic acids, could occur at these temperatures of uncertainty and shield the nucleic acid bases from the attack by the formaldehyde monomer. It is also possible that structural changes could be taking place in the spore at these temperature regions, possibly via changes in permeability, to interfere with the availability of formaldehyde at the reaction sites.

It was suspected that these irregularities in the "pseudo-Arrhenius" plots for the inactivation of the spores of the selected Bacillus strains by 0.5% w/v aqueous formaldehyde solution, could have serious practical implications if observed at LTSF treatments since the UK temperature for LTSF sterilization, 73°C ± 2°C, lies within this region of uncertainty. This phenomenon could also give an explanation for the random LTSF sterilization cycle failures often observed without any signs of mechanical failure. These observations could also help elucidate the mechanism of formaldehyde induced lethality in bacterial spores which is currently not very clearly understood.

It was considered necessary to establish whether the irregularities observed in response of the spores to inactivation by formaldehyde in aqueous solution, were observed when the spores were exposed to LTSF conditions. A commercial LTS/LTSF apparatus, the Miniclave 80, was modified by Line (229) to make it suitable for the investigation of the response of the spores to inactivation by LTSF at various defined conditions.

Spores of B. stearothermophilus NCIB 8224, produced on C-Ltd medium were selected to determine the influence of temperature and formaldehyde concentration on bacterial spore inactivation by LTSF using the modified apparatus. Data presented from these preliminary experiments revealed the need for additional modifications to the apparatus. Reduced reproducibility in the inactivation data was observed and bi-phasic inactivation kinetics were apparent in the survivor curves obtained. It was suspected that these observations could be due to variation in formaldehyde concentration in the chamber during the exposure period. While the modification allowed for accurate monitoring of the exposure temperature and pressure, it was not possible to monitor formaldehyde concentration during the exposure period. This is one of the reasons why biological indicators are necessary in LTSF sterilization and without an established biological indicator, the LTSF process cannot be fully assessed for its efficacy.

The fate of the administered formaldehyde in the chamber was then investigated indirectly by measuring the amount of

dissolved formaldehyde collecting in the effluent vessel. The method used was not expected to provide an absolute measurement of formaldehyde concentration at any time during the cycle, neither could it be expected to distinguish between different forms of formaldehyde present in the chamber. The method was however sufficient to demonstrate a definite formaldehyde concentration decay from the chamber at rates increasing with increased operation temperature. Other workers have also reported on decaying formaldehyde levels in LTSF sterilizers. The door of the modified apparatus was unheated and could have been responsible for the condensation that was observed during the operation of the LTSF cycle. This condensation was suspected of solubilizing some of the formaldehyde vapour and subsequently losing it through the chamber drain. There was therefore no means of checking the homogeneity of the chamber conditions.

The sensitivity of the test spores was observed to increase with increasing formaldehyde concentrations over the range 8.19 mg^{-1} (2.73×10^{-4} M) to 49.14 mg l^{-1} (1.64×10^{-3} M). The results show that enhanced sporicidal activity can be achieved by increasing formaldehyde concentrations in the chamber. However, consideration should be given to operator safety, risks of toxic residuals and mechanical failure due to possible polymerisation when deciding on the amount of formaldehyde for use in LTSF sterilization.

Temperature increases between 68°C and 73°C did not affect

the resistance of the test spores to inactivation by LTSF at 16.38 mg l^{-1} formaldehyde concentration. However, the resistance of the test spores to inactivation by LTSF was observed to increase as the temperature was increased from 75° to 80°C. The results were expected in the light of the observed temperature dependent decline in formaldehyde concentration during the operation of the LTSF cycle. It has been demonstrated that the amount of formaldehyde available for sterilization decreased as the operation temperature was increased. When the inactivation data obtained for the inactivation of the test spores to LTSF at 16.38 mg l^{-1} at temperatures between 68° and 80°C, was described by the Arrhenius relationship, there was no obvious linearity observed on the Arrhenius plot. There was however evidence of a positive slope which could be explained by the increasing resistance observed with increasing temperature. These investigations were preliminary and modifications to the apparatus are obviously required to monitor formaldehyde concentration accurately, to maintain the defined conditions and to reduce condensation. It is envisaged that after such modifications, significant inactivation in excess of 1 log-cycle can be obtained. The effects of temperature and formaldehyde concentration on the inactivation of spores by LTSF can then be invetigated using a more efficient apparatus.

The work described in this thesis has identified spores of B. stearothermophilus NCIB 8224, produced on C-Ltd medium as suitable for development as biological indicators for LTSF sterilization since the strain was demonstrated to meet most of the

requirements of an ideal biological indicator organism. Spores of this strain can be expected to exhibit high resistance to inactivation by LTSF with log-linear inactivation kinetics assuming of course that the mechanism of inactivation by LTSF is similar to the mechanism of inactivation by formaldehyde in aqueous suspension.

Suggestions for Future Work

If the Miniclave 80 LTS/LTSF sterilizer is to be of any valuable use as an experimental apparatus to recreate LTSF conditions likely to exist in commercial LTSF sterilizers, additional modifications are obviously necessary. Some of these additional modifications are currently being undertaken.

A formaldehyde input and measuring device, capable of administering multiple formaldehyde volumes in defined volumes and at defined frequency is obviously necessary to maintain set formaldehyde levels throughout the exposure period.

A heated door of the chamber would improve homogeneity of temperature in the chamber and discourage condensation and subsequent solubilization of vaporized formaldehyde on unheated surfaces. An accurate method to measure formaldehyde concentrations during the operational cycle is necessary to monitor this parameter of LTSF.

Modifications are also necessary to enable the apparatus to evacuate a dry chamber and to inject formaldehyde vapour pulses to heat it up to set conditions, before the hold conditions. A single formaldehyde injection at the beginning of the hold period followed by admission of dry saturated steam could then enable a comparative study of the relative efficiencies of the current formaldehyde sterilizing techniques (F/LTS and LTSF).

Attempts should be made to establish the minimum amount of formaldehyde required for sterilization, in order to reduce the formation of formaldehyde residues. A lower operating temperature than 73°C should be attempted to enable processing of more heat sensitive optical and electronic apparatus used in hospitals.

B. stearothermophilus NCIB 8224 spores produced on C-Ltd medium have been recommended as suitable for development as biological indicators for LTSF. More studies are necessary to determine their response to inactivation by LTSF using the fully modified Miniclave 80. A higher G.I. than 68% is desirable therefore sporulation medium composition should be studied to improve sporulation and germination index of the spore. Recovery conditions should also be optimised for better results.

Another approach for future research work on LTSF sterilization is to conduct studies to determine the nature of the sporicidal activity of formaldehyde and the optimum conditions in which it can take place.

APPENDICES

APPENDIX 1

A1. Gravimetric Analysis on the Precision Accuracy of Gilson Pipettes

A1.1 Gilson P200

Sample	20 µl	25 µl	100 µl	200 µl
1	0.200	0.0249	0.0972	0.2005
2	0.0200	0.0255	0.0975	0.2006
3	0.0202	0.0245	0.1000	0.2000
4	0.0204	0.0253	0.1007	0.2005
5	0.0203	0.0251	0.1004	0.2025
6	0.0210	0.0250	0.1003	0.0200
7	0.0199	0.0250	0.1005	0.1999
8	0.0202	0.0249	0.1018	0.2010
9	0.0201	0.0251	0.1004	0.2009
10	0.0200	0.0250	0.1006	0.2001
$\bar{x}$	0.02021	0.02503	0.09999	0.2006
S.D.	3.1780×10^{-4}	2.62679×10^{-4}	1.345×10^{-3}	7.70281×10^{-4}
C.V.	1.57%	1.05%	1.35%	0.38%
Error	1.23%	0.29%	0.16%	0.48%

S.D. = standard deviation of the statistical mean

$$\left(\frac{\Sigma (\bar{x}-x_i)^2}{n-1} \right)^{\frac{1}{2}}$$

$\bar{x}$ = mean statistical distribution

x_1 = individual volume (weight) delivered

n = number of sample units inspected

$$\text{Error (accuracy of mean)} = \frac{\text{wt at } 20°C - \bar{x}}{\text{wt at } 20°C} = \frac{0.99823 \times x_n \times 10^{-3} - \bar{x}}{0.99823 \times x_n \times 10^{-3}}$$

where x_n = nominal volume in µl.

A1.2 Gilson P1000

Sample	0.20 ml	0.50 ml	1.00 ml
1	0.2020	0.4987	0.9865
2	0.2025	0.5000	0.0909
3	0.2060	0.4985	0.9990
4	0.2015	0.4944	0.9895
5	0.2010	0.4899	0.9991
6	0.2029	0.4914	0.9897
7	0.2061	0.4986	0.9988
8	0.2032	0.4912	0.9889
9	0.2030	0.4976	0.9890
10	0.2000	0.4986	0.9953
$\bar{x}$	0.20282	0.49589	0.99256
S.D.	1.9674×10^{-3}	3.79281×10^{-3}	4.92887×10^{-3}
C.V.	0.9%	0.76%	0.49%
Error	1.58%	0.65%	0.57%

A1.3 Gilson P5000

Sample	4.5 ml	1 ml	5 ml
1	4.5135	0.9844	4.9899
2	4.4982	0.9899	4.9876
3	4.5038	0.9899	4.9915
4	4.5175	0.9899	4.9898
5	4.5109	0.9899	4.9901
6	4.5137	0.9969	4.9900
7	4.5184	0.9969	4.9900
8	4.5063	0.9880	4.9898
9	4.5071	0.9961	4.9963
10	4.5138	0.9907	4.9899
$\bar{x}$	4.51032	0.99212	4.99148
S.D.	6.38366×10^{-3}	4.66209×10^{-3}	3.71065×10^{-3}
C.V.	0.14%	0.47%	0.07%
Error	0.4%	0.6%	0.007%

APPENDIX II

STATISTICAL ANALYSIS

A2.1 Least Squares Regression Analysis

When a linear relationship is assumed to exist between two variables it is usual to fit a straight line by least squares regression analysis. The simplest statistical model for this assumes that the independent variable (x) is known without error of measurement and that the corresponding measured values of the dependent variable (y) are scattered normally from their true values. Hence each value of y_i of the dependent is normally distributed about the mean, with a variance σ^2.

The method of least squares obtains estimates of c and m in the equation $y = mx + c$ such that the sum of the squares of the deviations of the observations y_i from their mean is a minimum.

These values are:-

$$m = \frac{n\Sigma x_i y_i - \Sigma x_1 \Sigma y_1}{n\Sigma x_1^2 - [\Sigma x_1]^2}$$

$$= \frac{\Sigma (x_i - \bar{x})\ (y_i - \bar{y})}{\Sigma (x_i - \bar{x})^2}$$

$$c = \frac{\Sigma y_i - m\Sigma x_i}{n}$$

$$= \bar{y} - m\bar{x}$$

where n is the number of points on the line.

Variance of the Slope (m)

This is termed s^2_m and is given by the equation

$$s_m^2 = \frac{\sigma_e^2}{\Sigma(x_i-\bar{x})^2}$$

where σ^2_e is the residual variance of the dependent variable y and may be obtained by dividing the residual sum of squares ΣD^2 by (n-2) were

$$\Sigma D^2 = \Sigma(y_i-\bar{y})^2 - \frac{\left[\Sigma(x_i-\bar{x})(y_i-\bar{y})\right]^2}{\Sigma(x_i-\bar{x})^2}$$

Residual sum of squares (n-2) degrees of freedom	Total sum of squares (n-1) degrees of freedom	Sum of squares due to regression 1 degree of freedom

$$= \Sigma(y_i-\bar{y})^2 - m^2\,\Sigma(x_i-\bar{x})^2$$

The denominator (n-2) shows that two degrees of freedom have been lost because both the slope and the intercept were estimated from the data. The standard deviation of the slope is given by the square root of the variance.

Variance of the Intercept (c)

This is termed: $$s^2_c = \frac{\Sigma x_i^2 \sigma_e^2}{n\Sigma(x_i-\bar{x})^2}$$

where $$\sigma^2_e = \frac{\Sigma D^2}{(n-2)}$$

The standard deviation of the intercept is given by the square root of the variance.

A2.2 To Determine the Equality of Two Estimates of a Parameter (Student's t-test)

The equality of estimates p_1 and p_2, with respective variances s_1^2 and s_2^2, of a parameter p is assessed as follows:-

$$t = \frac{p_1-p_2}{(s^2_1 + s^2_2)^{\frac{1}{2}}}$$

The value of t is compared with tabulated values with $n_1 + n_2 - 4$ degrees of freedom, where n_1 and n_2 are the numbers of observations used in the estimation of p_1 and p_2 respectively. If the value of $t(t_{calc})$ does not exceed the tabulated value (t_{tab}) at the 5

percent (p = 0.05) probability level, the values are assumed to be indistinguishable at this level.

A2.3 To Determine the Equality of Two Means of a Parameter (Student's t-test)

The equality of means x_1 and x_2, with respective variances s^2_1 and s^2_2, of a parameter is assessed as follows:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s(\frac{1}{n_1} + \frac{1}{n_2})^{\frac{1}{2}}}$$

where n_1 and n_2 are the number of observations used in the estimation of x_1 and x_2. s represents the total standard deviation given by:-

$$s = \left[\frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{(n_1 + n_2) - 2}\right]^{\frac{1}{2}}$$

The value of t is compared with tabulated values with $n_1 + n_2 - 2$ degrees of freedom. If the value of $t(t_{calc})$ exceeds the tabulated value (t_{tab}) at the 5 percent (p = 0.05) probability level, the means are considered to be significantly different at that level.

A2.4 To Determine the Equality of More Than Two Means of a Parameter (Analysis of Variance)

Where the data is separated into a series of groups, each

consisting of a number of observations or measurements, analysis of variance and an F test is used to establish if the variance between the groups is significantly greater than the variance within the groups. The following quantities are calculated from the data:-

A) Uncorrected Sum of Squares

$$= \sum_{i=1}^{k} \sum_{j=1}^{n} x_{ij}^2$$

where k is the number of groups, n is the number of observations in each group and x^2 is the square of the observation or measurement.

B) Mean Sum of Squares of Group Totals

$$= \sum_{i=1}^{k} \frac{\left(\sum_{j=1}^{n} x_{ij}\right)^2}{n_1}$$

C) Mean Sum of Squares of Observations

$$= \frac{\left[\sum_{i=1}^{k} \sum_{j=1}^{n} x_{ij}\right]^2}{N}$$

where N is the total number of observations.

An Analysis of Variance table is now set up:

	Sum of Squares	Degrees of Freedom	Variance
Between Groups	B-C	k-1	$\frac{B-C}{k-1} = s_2^2$
Within Gups	A-B	n-1	$\frac{A-B}{N-k} = s_1^2$
Residual	By subtraction	(k-1)(n-1)	
Total	A-C	N-1	$\frac{A-C}{N-1}$

The ratio s_2^2/s_1^2 will come from an F distribution and if the group are not significantly different this calculation value of F will be less than that tabulated at p = 0.05 with k-1 and N-k degrees of freedom.

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